THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

SCIENCE AND MEDICINE DIVISION

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

Editors :

C. L. EVANS
P. FILDES
W. E. GYE
E. H. KETTLE
J. McINTOSH
H. MACLEAN
J. A. MURRAY
J. B. ORR
W. J. TULLOCH
C. M. WILSON

VOLUME TWO

1921

LONDON : H. K. LEWIS & CO. LTD., 136, GOWER STREET, W.C. 1.
EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C. 1.
CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

> "I lay it down as a maxim, that no man can establish or support a reputation in any branch of experimental philosophy, such as shall really descend to posterity, otherwise than by experiment; and that commonly the more a man works, and the less he writes the better, at least the more valuable will be his reputation."
>
> Arthur Young. *Travels in France.*

LIBRARY
AUG 23 1965
UNIVERSITY OF TORONTO

RB
125
J67
v. 2
Cop. 2

1001700

CONTENTS

No. 1.—FEBRUARY.

PAGE

Hill, Leonard, and McQueen, James M.—The Measurement of Capillary Blood-Pressure in Man 1

Maitland, H. B., Cowan, Mary L., and Detweiler, H. K.—Spontaneous and Artificial Pulmonary Lesions in Guinea-Pigs, Rabbits and Mice 8

Fildes, Paul.—The Nature of the Effect of Blood-Pigment upon the Growth of *B. influenzæ*. 16

Schütze, H.—Hæmagglutination and its Medico-Legal Bearing, with Observations upon the Theory of Isoagglutinins 26

Ponder, Eric.—The Presence of Hæmolytic Substances in Human Urine 34

Taniguchi, Tenji.—The Sachs-Georgi Syphilis Reaction and its Relation to the Wassermann Reaction 41

No. 2.—APRIL.

Douglas, S. R.—The Question of Serological Races of *V. choleræ* and the Relation of some other Vibrios to this Species . . 49

Bennett, T. Izod, and Dodds, E. C.—The Gastric and Respiratory Response to Meals 58

Alexander, W.—An Inquiry into the Distribution of the Blood Groups in Patients Suffering from "Malignant Disease." . . . 66

Pannett, C. A., and Wilson, C. M.—The Influence of Bile-Salts upon Gastric Function 70

Noël Paton, D., and Watson, A.—The Ætiology of Rickets: An Experimental Investigation 75

Browning, C. H., and Gulbransen, R.—The Antiseptic Potency of Acriflavine, with Considerations on the Variability of Results in Testing Antiseptics. 95

Jensen, Vilh.—On Picro-Carmine 103

No. 3.—JUNE.

PAGE

Evans, C. Lovatt.—The Reaction of the Blood in Secondary Anæmia . 105

Yorke, Warrington, and Macfie, J. W. S.—The Mechanism of Autolysis in Paroxysmal Hæmoglobinuria 115

Douglas, S. R., and Fleming, Alexander.—On the Antigenic Properties of Acetone-Extracted Bacteria 131

Macnalty, A. Salusbury.—The Morbid Histology, Bacteriology, and Experimental Pathology of *Encephalitis lethargica* . . 141

Fildes, Paul, and McIntosh, James.—An Improved Form of McIntosh and Fildes' Anaërobic Jar 153

Jensen, Vilh.—A New Immersion Fluid 155

No. 4.—AUGUST.

Andrewes, F. W., and Neave, Sheffield.—The Nature and Systematic Position of *B. paratyphosus C* 157

Douglas, S. R.—On Some Characters of the Cleavage Products of Certain Bacteria, with Special Reference to their Toxicity and Antigenic Properties 175

Ponder, E.—The Occurrence of Hæmolytic Substances in Normal Urine. 192

Onohara, K.—The Physico-Chemical State of Sugar in the Blood . 194

Yabe, S.—Grouping of Influenza Bacilli 197

No. 5.—OCTOBER.

Hill, Leonard, and McQueen, James.—Capillary Blood-Pressure and the Glomerular Filtration Theory 205

Barratt, J. O. Wakelin.—The Theory of Agglutinin Estimation . 214

Yabe, S.—The Preventive Effect of Influenza Vaccine . . . 223

Pratt-Johnson, J.—Comparative Immunity Tests with Saline Vaccines and Lipovaccines 232

Pratt-Johnson, J.—Further Studies on the Production of Immunity in Rabbits Against an Organism of High Virulence for the Species 239

Hall, I. Walker, and Fraser, A. D.—The pH Range for Staphylococci 242

No. 6.—DECEMBER.

PAGE

Buchanan, J. Arthur, and Higley, Edith T.—The Relationship of Blood-Groups to Disease 247

Kanai, S.—Dysentery Immunisation in Rabbits by the Oral and Subcutaneous Methods 256

Wolf, C. G. L.—The Comparative Influence of Pure and Commercial Sugars and of Combined and Separate Sterilisation on Bacterial Metabolism 266

Benians, T. H. C.—Septicæmia: The Selective Deposition of the Colon Typhoid Group of Bacteria in Fixation Abscesses 276

Maitland, H. B., and Cameron, Gordon C.—A Study of Hæmoglobinophilic Bacteria by Agglutination and Agglutinin Absorption . 283

Ponder, Eric.—The Relation between Bile Salts and Hæmolysis in the Blood-Stream 289

Index 292

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

THE MEASUREMENT OF CAPILLARY BLOOD-PRESSURE IN MAN.

LEONARD HILL, F.R.S.,

National Institute for Medical Research, Hampstead, London;

AND

JAMES M. McQUEEN.

Received for publication December 17th, 1920.

An accurate measurement of capillary blood-pressure in man is much to be desired, but difficult of attainment. It is true that measurements are to be found in every text-book, and equally true that these measurements are tacitly, at least, applied as accurate to other physiological problems, such as the origin and production of lymph. Physiologists have not hesitated to accept the current measurements, correlated them with measurements of the osmotic pressure of colloid, such as blood-plasma, and found a balance of pressure in the capillaries in favour of filtration or other purely physical theory of the origin of lymph. No less, in the theories of the production of urine, capillary blood-pressure measurements are made of fundamental importance.

Measurements of capillary blood-pressure in man were first attempted by V. Kries, using a glass plate on the terminal phalanx of the finger below the finger-nail. By placing weights on a pan attached to the plate, and calculating the surface area of the plate and the weight which just sufficed to blanch the area below the glass, measurements of capillary blood-pressure varying, of course, at different levels from the heart were obtained ranging from 30–40 mm. Hg. and over.

It was assumed in these measurements that the area comprised a more or less uniform capillary field, and that the resistance of the skin was not a factor of much importance.

It is unnecessary to enter into the details of other methods of achieving capillary blood-pressure measurements in man, as the data, no matter what method is employed, do not materially differ from those given by V. Kries. Thus, recently, Danzer and Hooker (1) have microscopically observed the flow in the skin adjacent to the nail, using a suitable chamber into which air was pumped so as to stop the flow, and a manometer to measure the pressure. They found in eighteen individuals the stopping-pressure averaged 18–26·5 mm. Hg.

It was, however, shown by one of us (L. H.) (2) that if a hair be taken which will just blanch the skin when pressed on it till it bends, a weaker hair will suffice after shaving off the outer horny layers of the epidermis.

The difficulty in securing a uniform capillary field in man on which observations might be made, it is safe to say, will hardly be overcome. The arrangement of arterioles, capillaries and venules as a meshwork prevents the absolute isolation of any component of the mesh. In the case of the frog's web an area of 2 mm. in diameter includes all these vessels. On compression of such an area blanching occurs only when the systolic pressure in the arterioles supplying the area is overcome, as may be seen by observation of the frog's web compressed by Roy and Graham Brown's method (3). The wider the area chosen the larger may be the arterioles compressed and the higher may the readings be. Accordingly a method of compression should be chosen so that a minimal area is compressed. Further, if the compression could be made momentary, it might express blood and blanch the area before the pressure had time to bank up to the systolic pressure in the arteries which supply the arterioles of the compressed part.

Observations made by one of us (L. H.) (4) on the frog's web and mouse's ear and mesentery show that 2–5 cm. of water is the pressure which, when quickly applied, momentarily checks the flow in the arterioles and their main capillary branches, and that the checking and not the stopping pressure, which may be 35 cm. of water, gives the true index. Unfortunately it is not possible to secure such a momentary index of compression in man, so the readings are bound to be too high. The venous pressure in the main veins of the arm as measured by observing the collapse of the superficial veins on raising the arm above the heart-level is some 3–10 cm. of water. Now the venule-pressure closely approximates to that in the capillaries in the case of the frog and mouse.

Some twenty-five years ago the capillary pressure in the brain of the dog was measured by one of us (L. H.) (5) by finding the pressure required just to flatten that part of the brain which bulged out into a trephine hole. A tube closed by a membrane was screwed into the trephine hole so that the membrane came into contact with the brain. The tube was filled with water and connected with a glass tube which contained an air-bubble index, and this in its turn with a manometer and pressure bottle. The air index was forced out from the zero position by the brain, was brought back to this position by raising the pressure bottle and the pressure indicated by the manometer then read. This method was applied (in the case of a man) to a trephine hole covered with skin, a special contrivance being used to secure an air-tight connection of the apparatus with the shaved skin in which the principle of the cupping glass was used. The capillary pressure in the brain, with the man in the horizontal position, by such a method was found to be a very few millimetres of mercury. It was in the dog found to be the same as the pressure in the venous sinuses and as that of the cerebro-spinal fluid. It varied with the rise of venous pressure due to expiratory effort, with rise of arterial pressure and with change of posture. In the erect posture it might be at times zero or even negative. In the infant exhausted by illness the anterior fontanelle is depressed. On the other hand, in a fit of crying in the case of a lusty infant it bulges out and feels tense. In the spasm produced by strychnine, or essential oil of absinthe, the pressure in the capillary vessels, which is the same as the pressure of the brain against the skull-wall, might rise to 40–50 mm. Hg., owing to the great increase of arterial pressure, coupled with an obstruction of the venous return. As has already been stated, the capillary pressure of the brain was found to be the same as that of the cerebro-spinal fluid. In a case of hydrocephalus the pressure varied with the compression of the head by the observer's hands and became zero when the fluid was aspirated off.

So, too, in the case of the eye, wherein the capillary pressure is regulated by the secretion pressure of the aqueous fluid, the pressure of the fluid outside and the blood inside the capillaries always balance one another. Thus it was shown by Hill and Flack (6) that on raising the pressure in the aqueous chamber the circulation in the eye continued, as shown by the outflow from a vortex vein, until the arterial pressure (measured in the carotid) was just overtopped, *i. e.* the pressure in the capillaries and veins rose *pāri passu* with that of the fluid in the aqueous chamber. It was also shown that when the aqueous fluid was allowed to escape the iris bulged forward owing to the pressure in the capillaries no longer being balanced; in such a case a compression of the abdomen, through raising the venous pressure, easily burst these vessels in the iris and caused hæmorrhage. Struggling during an operation on the anterior chamber of the eye may have the like effect on man.

In the case of the salivary gland of the dog, Hill and Flack (7) also showed that when the secretory pressure rises above the arterial pressure, the outflow from the salivary vein continues. They pointed out that the cells of each alveolus are enclosed by a *membrana propria* which limits their expansion, so that the venules can be compressed, but not shut up as the secretory pressure rises. Owing to arterial dilatation and compression of the veins and venules by the swollen alveoli the pressure rises in the veins and capillaries, and the blood-flow becomes a rapid one through the whole vascular system of the gland, which is approximate to a rigid system. The tenseness of the whole gland can then be felt by the finger. Similarly in inflamed parts the tension and throbbing are due not only to arterial dilatation, but also to the rise of imbibition pressure within, and swelling of the tissue cells, with consequent compression of veins, this in its turn leading to the vascular pressure in the inflamed part approximating throughout capillaries and venules to arterial pressure. The sequence of the phenomena is exactly similar to that which occurs when the arm is compressed by the cuff of the sphygmomanometer, a sequence which may be observed under the microscope in the frog's web, lung, etc., when compressed by the membrane of an apparatus similar to that devised by Roy and Graham Brown for measurement of capillary pressure.

In the case of man, Hill and Flack (7) found that it took but a very few centimetres of water-pressure to drive fluid under the skin through a hypodermic needle, and the conclusion was reached that capillaries must be exsanguined when water is so driven in, and that the pressure required to effect this is very low under normal resting conditions. When the arm is compressed by the cuff of the sphygmomanometer with a pressure just below that in the artery, the pressure in the superficial veins rises up to this pressure (7), but it does not then take anything like this pressure to drive water under the skin of the arm. The veins are filled through the wider anastomoses while many capillaries are still largely not filled to congestion. In a case of œdema of the leg a hollow needle indicated a pressure of a few centimetres of water when the leg was placed at the same level as the heart, Compression of the leg by the hands at once raised the pressure, and it is obvious that capillary pressure must rise *pari passu* with that of the œdema fluid for the circulation to continue. The conclusion was reached that the capillary pressure is largely governed by the imbibition pressure of the tissue-cells, vessels and cells being both confined by the membranes and skin of the body.

In the eye the secretory pressure of the aqueous fluid regulated by the cells of the ciliary processes fixes the pressure at some 25 mm. Hg., while in the brain the secretory pressure of the cells of the choroidal fringes fixes the pressure at some 5 mm. Hg., the subject being in the horizontal position and resting. With rise and fall of venous, arterial or secretory pressure, tissue fluid and capillary pressures vary together.

In the skin of the healthy person the pressures are kept just positive, giving the fulness to the countenance. The face looks drawn when the imbibition tone of the tissues falls and the circulation fails to keep the capillaries distended, *e.g.* in acute diarrhœa.

To try and arrive at a juster measure of the pressure in the capillaries in man, one of us (J. McQ.) has designed the following method :

If water from a jet fed from a reservoir kept at constant pressure-head be played on the skin below the nail or on the skin covering the distal end of the metacarpal bone of the thumb, the jet having a 2 to 2·5 mm. bore diameter, it will be noted that if the pressure be sufficiently high, the area impinged on can be rendered white ; when the jet is removed a red blush of returning blood is seen. The horizontal jet of water striking the thumb being dispersed by impact tends to reflect light, and if we look into the jet the skin below appears whitened ; such whitening must not be taken as the index. Moving the jet quickly to one side and watching the area just impinged on by the jet of water we note the return of the blood into that blanched area. The pressure which just suffices to produce blanching is the first measurement to be obtained. That pressure can be easily calculated. Thus, let the jet be turned so as to throw its column of fluid into the air alongside a meter scale ; the distance of the upward throw of water is obviously the pressure of impact on the thumb in centimetres or millimetres of water. The jet is kept less than half an inch from the thumb, so that a small deduction from the scale reading can be allowed for that distance. A fairly accurate measurement is thus achieved of the pressure necessary to blanch a 2 to 2·5 mm. of skin area. The water should be kept at body-heat to prevent cooling of the vascular area.

When readings of pressure are taken at heart level by this method, they do not materially differ from the readings of pressure obtained by V. Kries. But be it noted, as the flow is stopped in the blanched part the readings are of arteriole, not capillary pressure.

Moreover, there remains to be considered the resistance of the convex horny layer of skin to compression. There are also the resistance of the vascular wall to compression, the friction of the blood as it is expelled from the compressed area, the resistance of the lymph or tissue fluid surrounding the capillaries. In the frog's web these resistances are very small. One of us (L. H.) has shown that a pressure of 1 cm. of water applied to the membrane of the Roy and Graham Brown apparatus is sufficient to drive the corpuscles quickly along the vessels of the web wherein the circulation has been arrested. Up to the present no adequate method has been devised for estimating these factors and the resistance of the horny layer of the skin in man. Their sum can be accurately estimated provided one can reduce the blood-pressure in the compressed area to zero. Then the pressure taken to blanch the area is a measure of the resistance factors.

To reduce the blood-pressure to zero is not an insoluble problem. It is known that the veins of the dorsum of the hand at or below the heart level can be seen to bulge. The lower the hand is placed, the tenser the veins become. Now if the hand is raised above the heart level, at a certain point the veins collapse, because the venous pressure has been overtopped by gravity. The venous pressure in the hand is now zero. If the hand be raised still higher and the brachial artery occluded, the arterial pressure entering the hand is in its turn reduced to zero. Accordingly the capillary pressure becomes zero. A tourniquet of rubber tubing can be used or the cuff of the sphygmomanometer connected with a compressed air cylinder so as to secure a rapid application of pressure to the artery. The pulse is found to be absent in the radial artery, and if we blanch a large area of skin by compression the area remains white. This in itself is a proof of zero pressure. If an elastic area is compressed there is, of course, a recoil effect on removal of compression which tends to suck the blood back into the area. This is seen notably in the 2 to 2·5 mm. area compressed by the jet. The smaller the area compressed, the greater the recoil effect.

Had the blood in the area been endowed with a pressure, then the large blanched area would quickly blush once more. Further, in time the blood tends to pass by gravity or muscular movements to the interior of the thumb or into big superficial veins, which is in itself another proof of the absence of arteriole pressure in the area. The phenomenon of discoloration in dependent parts in the dying patient or in the dead is analogous. In order to get enough blood in the capillaries to give a clear index, it is advisable to put a rubber band round the wrist before elevating the arm and compressing the artery, and then to remove this band.

If the jet be projected on the thumb when the area has been thus brought to zero pressure, a measure of the pressure in centimetres or millimetres of water required to blanch the area is now obtained. This pressure measurement—the measurement of resistance factors—has to be subtracted from the original measurement obtained when the normal conditions of pressure pertained in the arterioles.

The diminished reading is now the actual arteriole pressure.

To cite examples:

Subject E.—Hand at heart level. Pressure to blanch 2·5 mm. area = 35 cm. water. Resistance measurement (after vascular area reduced to zero pressure) = 24 cm. water. Difference = arteriole pressure = 11 cm. water.

The readings for this subject on other occasions were: 45 and 31; difference = 14 cm. water. 41 and 31; difference = 10 cm. water.

Subject M.—The readings for subject M were 43 and 31; difference = 12 cm. On another occasion, 50 and 35; difference = 15 cm.

Subject W.—The readings for subject W were 40 and 26; difference = 14 cm. water.

The arteriole pressure at heart level comes out then as about 10 mm. Hg., and this is too high a reading because the method of compression is not momentary enough and allows time for banking up. The observations made by one of us (L. H.), using the Roy and Graham Brown method, show that the capillary pressure in the mouse's ear and mesentery is not more than 2–3 cm. of water—about 2 mm. Hg. The deductions, then, that have to be made from all the usual readings that are in vogue for capillary blood-pressure are important, and must be reckoned with in consideration of physical theories of lymph production.

The physical theory of the production of urine which postulates a glomerular capillary blood-pressure amounting to a figure as high as 20 per cent. below carotid blood-pressure also requires to be modified very materially. One of us (L. H.) has for many years insisted that the supposed filtration pressures do not and cannot exist because the tissue fluid pressure balances the capillary pressure. This is seen to be so in the case of the brain and eye. The acceptance of the low value of capillary pressure makes the production of capillary stasis in disease generally, or in peripheral parts, more intelligible. Thus the hands of a dying patient may show obvious signs of capillary stasis while the pulse is still quite palpable. But if the arteriole pressure be normally as low as 5 mm. Hg. any failure of the force of the heart-beat will, by sending the pressure lower, produce the condition of stasis. There is in the capillary field little margin of safety.

SUMMARY.

That pressure which stops the flow through and expresses blood from the skin has been generally taken as signifying the capillary blood-pressure. In truth it indicates the pressure in the small arteries which feed the compressed part after an allowance has been made for error arising from the pressure required to deform the convex horny layer of the skin. The method devised measures this error and arrives at the true pressure in the small arteries. The pressure of a small jet of water is found which will just blanch the skin of the hand held at heart level (1) in the normal condition, and (2) with the capillaries at zero pressure after the veins of the forearm have been emptied by gravity and the brachial artery occluded by a tourniquet. When (2) is deducted from (1) a reading of about 10 mm. Hg. is obtained. Reference is

made to previous measurements of capillary pressure in the brain, etc., and to recent experiments carried out on transparent membranes of frogs, mice, etc., by one of the authors (L. H.). These show that a momentary compression equal to 2–5 cm. water suffices momentarily to check the flow of corpuscles in the arterioles or its main capillary branches. The true capillary pressure as indicated by these figures is a very low one, and theories of lymph formation, etc., based on a capillary pressure of some 30 mm. Hg. must be modified accordingly. The relation of the swelling of the tissue cells to the capillary circulation in inflammation is discussed, also the relation of the pressure of the cerebro-spinal fluid, aqueous humour and salivary secretion.

REFERENCES.

1. Danzer, C. S., and Hooker, D. R.—"Determination of the Capillary Blood-pressure in Man with the Micro-capillary Tonometer," *Amer. J. Physiol.*, 1920, **52**, 136.
2. Hill, L.—'Further Advances in Physiology,' London, 1909 (Arnold), p. 149. "Blood-vessels and Pressure," *Lancet*, 1920, **1**, 359.
3. Roy, C. S., and Brown, J. G.—"The Blood-pressure and its Variations in the Arterioles, Capillaries and Smaller Veins." *J. Physiol.*, 1879-80, **2**, 323.
4. Hill, L.—"The Capillary Blood-pressure," *J. Physiol.*, 1920, **54**, xxiv.
5. Hill, L.—'The Physiology and Pathology of the Cerebral Circulation,' London, 1896 (Churchill). "The Experimental Pathology of the Cerebral Circulation," Allbutt and Rolleston's *System of Medicine*, London, 1910, **8**, 3 (Macmillan).
6. Hill, L., and Flack, M.—"The Secretion of the Aqueous and the Intra-ocular Pressure," *Proc. R. Soc.*, B., 1912, **85**, 439.
7. Hill, L., and Flack, M.—"The Salivary Secretion," *ibid.*, 1912, **85**, 312.

SPONTANEOUS AND ARTIFICIAL PULMONARY LESIONS IN GUINEA-PIGS, RABBITS AND MICE.

H. B. MAITLAND, M.B., MARY L. COWAN, B.A., M.B.,
AND H. K. DETWEILER, M.D.

From the Department of Pathology and Bacteriology, and the Department of Medicine in the University of Toronto.

Forwarded for publication October 25th, 1920.

INVESTIGATIONS made in searching for a filter-passing virus as a possible factor in the ætiology of epidemic influenza have been reported in a former paper.(1) No evidence was found to support such a possibility, but in the animals used for the experiments, particularly the guinea-pigs, two types of pulmonary lesions, hæmorrhagic and proliferative, were observed. It was shown that these lung changes did not indicate experimental influenza. They were believed not to be the result of the inoculation of Berkefeld filtrates of material from influenza patients—sterile in ordinary culture—which the animals received, because a similar percentage of lesions occurred in animals inoculated with control material, and also in untreated animals from the stock pens.

The hæmorrhage and the proliferation were distinctly different types of abnormality. Each was observed alone in many cases. When they occurred together the association was of no fundamental significance, neither being dependent on the other. Therefore they were considered to have different origins. In order to determine, if possible, how they were produced, the experiments to be reported in this paper were performed.

THE HÆMORRHAGIC TYPE OF LESION.

Apart from the papers relating to the ætiology of influenza, this striking pulmonary lesion does not appear to have received attention in the literature. Since this paper is specially concerned with it, a recapitulation of a former description (1) may be permitted. This lesion was most marked in guinea-pigs, and the following description refers to them.

On the surface of the lung the hæmorrhagic area had various forms. Sometimes the whole lung was thickly spotted with small hæmorrhages 2 to 3 mm. in diameter, and on section they were seen to be deep in the lung-tissue as well. In other instances solid hæmorrhagic lesions appeared on the surface in irregular-shaped areas, from 3 or 4 mm. to 8 or 10 mm. in diameter. These usually had a dark red centre and a brighter red periphery. Occasionally the greater part of one lobe, or of several lobes, was irregularly involved. In rabbits the lesion had exactly the same appearance, but was less extensive. It occurred also in mice.

Microscopically the lesion was characterised by several peculiar features. There was extensive extravasation of erythrocytes into the alveoli and even into the bronchioles. In the most extreme degree the air-holding tissue in a focus of hæmorrhage was almost obliterated, and only a few empty alveoli could be seen, isolated from one another, in a mass of red cells. In places there was less lung-tissue involved, and the hæmorrhage was more diffuse, the type of change being essentially the same, differing only in extent. There was also hæmorrhage into the alveolar walls, and engorgement of the capillaries. The blood-cells were not deformed, and stained evenly and well. Hæmosiderin was not present. One of the most striking features was the absence of leucocytic reaction.

After it had been established that this lesion did not result from inoculation (1) it became apparent that, in general terms, the cause must be some agent acting during life—infection, protein poison, etc.—or, on the other hand, some agonal phenomenon due possibly to the method of killing which had been adopted in the experiments. Against the view that the hæmorrhages were present in the guinea-pigs during life there were several arguments. The animals in which hæmorrhagic lungs were found—even the most extensive—did not appear to be ill. Their temperature was normal. There was no free fluid, or fibrin in the pleural sac. The blood cells were not degenerated. If they had been outside the vessels for any considerable time prior to death one would have expected to see some signs of their disintegration, and especially to have found some hæmosiderin. Neither was observed. Further, although foci of hæmorrhage may be a frequent result of infection, or of inoculation with pathogenic micro-organisms, in such instances one expects to find an associated local leucocytic reaction. This was entirely absent in the lesion under discussion, and the fact was so remarkable that in conjunction with the other features that have just been mentioned it seemed very improbable that the hæmorrhage could have resulted from an infection. The correctness of this deduction was confirmed by the results of culturing the lungs. The aërobic cultures in glucose ascitic broth made from about 150 hæmorrhagic guinea-pig lungs were almost always sterile. The few exceptions formed such a small percentage of the total that they did not detract from the value of this evidence.

The other alternative, then, was to consider the method of killing and the mode of death.

The routine method had been to hit the guinea-pig a blow on the back of the head. It became unconscious immediately. There was usually some tonic contraction of the muscles and a few gasping respirations before movement ceased and complete relaxation obtained. For the succeeding five minutes or more, during preparation for the autopsy, the heart continued to beat, making a quite forcible impulse on the chest-wall. Cessation of all functions was therefore neither immediate nor simultaneous.

It was thought that the only way in which the question of the presence or absence of these hæmorrhagic lesions during life could be definitely settled would be actually *to see* the lungs before any changes could possibly take place as the result of killing the animal. For this reason the following experiment was made in the hope of obtaining the lungs unaltered by any agonal phenomena which might have occurred in our previous work.

Experiment I.—Sixteen guinea-pigs were killed by cutting through the anterior thoracic wall and into the heart with one snip of the scissors. The chest was then immediately opened more widely and the diaphragm incised. Thus respiratory movements had no effect on the lungs, the circulation through them was destroyed, and the possibility of agonal lesions was reduced to a minimum. The lungs were removed and examined without delay.

All of these lungs were free from the hæmorrhagic lesion. In two there were, however, two or three superficial bright red flecks of hæmorrhage, situated on different lobes. They were about 2 to 3 mm. in diameter, and were so evidently recent, so isolated and small that no one could possibly mistake them for experimentally produced disease. Apart from these the gross appearance was quite normal.

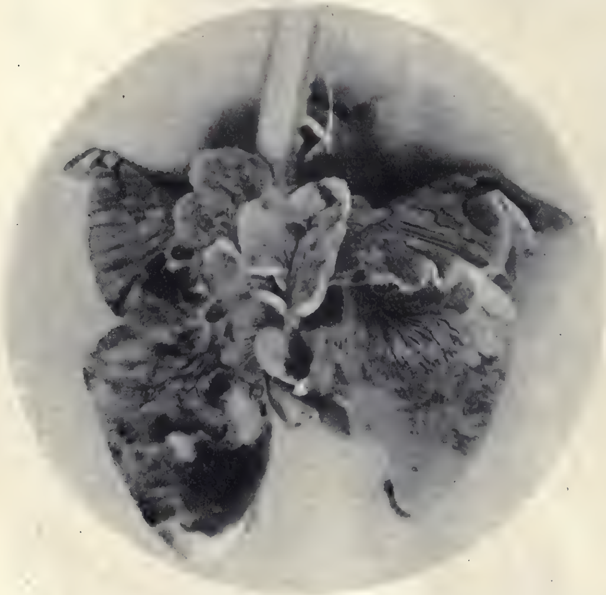


FIG. 1.—Lung of guinea-pig (*Experiment II*), killed by a blow on the head. Areas of hæmorrhage are present on two large lobes and one small lobe.

Two other guinea-pigs were treated in a similar way except that the cut which opened the thorax did not penetrate the heart, so that this was not opened until about one minute later. The lungs from each showed hæmorrhages. The first one had several areas, 6 to 8 mm. in diameter, involving parts of two lobes. The colour on removal was bright red, but in a few minutes the central part became much darker. On section it extended solidly into the lung—in other words it was a typical hæmorrhagic lesion. The second lung was similar.

Experiment II.—In order to compare accurately the effects of the different methods of killing, seventeen pigs from the same pen, raised under the same conditions, were killed by a sharp blow on the back of the head. This was the method used as a routine in the influenza experiments.

After the animal had been struck respirations sometimes ceased immediately, but usually continued for one or two minutes. The heart beat forcibly for five to eight minutes longer and a marked impulse could be felt on the chest-wall. When it became very faint the thorax was opened and the lungs removed. This length of time corresponded to the time occupied in sterilising the skin and preparing for the autopsy in the former experiments.

Six of these lungs had typical hæmorrhages (Fig. 1), the lesions corresponding in every way to those found during the influenza investigations. The result contrasts markedly with that of *Experiment I*. The only way to account for the presence of the hæmorrhages in the second experiment and their absence in the first was to assign their cause to the difference in the terminal state of the guinea-pigs. In the first experiment respiration and circulation ceased together. In the second the heart continued to beat forcibly for about five minutes after respiration had stopped. The guinea-pigs used for the two experiments were similar. Some were killed alternately, first by one method and then by the other ; some were killed by one method or the other in batches on successive days.

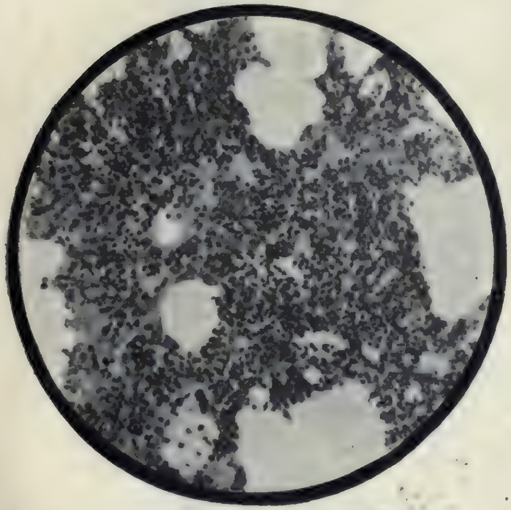


FIG. 2.—Section of lung in *Fig.* 1, showing area of hæmorrhage of moderate extent. (× 80.)

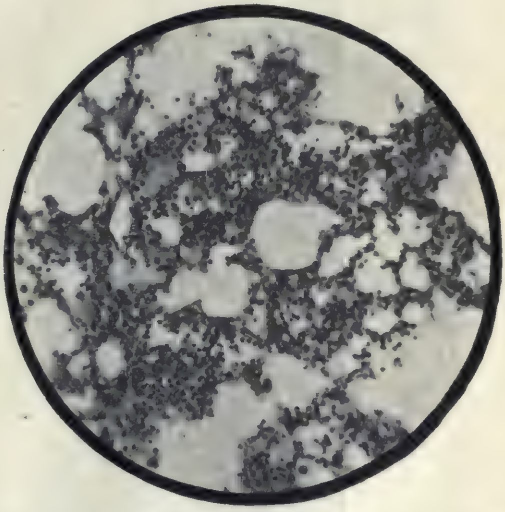


FIG. 3.—Section of lung in *Fig.* 1, showing a small amount of diffuse hæmorrhage. (× 80).

The results of *Experiment I* corroborated the evidence previously discussed, supporting the view that the hæmorrhages were not present in the lungs during life, and showed that by employing a suitable method of killing they could be avoided. They were, therefore, an agonal phenomenon. Sixteen is a comparatively small series, but in no other series of equal size examined by us have hæmorrhages failed to appear, and therefore this conclusion seems justifiable.

The practical importance of this lesion is, of course, the relation it has to the experimental pathology of the guinea-pig's lung. In some circumstances the method of killing used in *Experiment I* might be a satisfactory procedure, but it has the disadvantage of preventing the making of cultures from the thorax, and since this is often important, a third method was tried in order to overcome this difficulty.

Experiment III.—Twelve guinea-pigs from the same pen as the others were killed by rapid chloroform anæsthesia. The chloroform was poured on absorbent cotton in the bottom of a beaker in which the animal's head and shoulders were placed. Respiration ceased in from $1\frac{1}{4}$ to $3\frac{3}{4}$ minutes. The force of the cardiac impulse rapidly weakened during that time, and when breathing stopped it was barely perceptible, and sometimes could not be felt. The thorax was then opened immediately.

Ten lungs were free from typical hæmorrhage but one of them had some small isolated flecks.

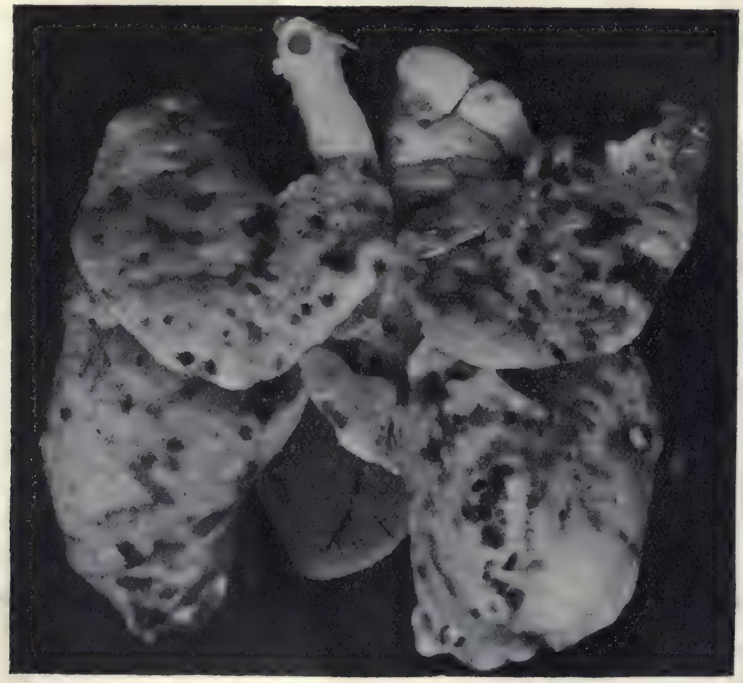


Fig. 4.—Lung of guinea-pig (*Experiment III*), killed by rapid chloroform anæsthesia. Diffuse distribution of small areas of hæmorrhage, some of which coalesce to form larger lesions.

One lung was evidently diseased. About half of one lobe was shrunken, smooth, firm and dull salmon-red in colour. Similar tissue was scattered throughout in very small patches, giving the surface a speckled appearance.

The twelfth lung was riddled with hæmorrhages from 2 to 6 mm. in diameter, some coalescing and forming irregular blotches on the surface—an extensive typical lesion (Fig. 4).

Thus killing by chloroform does not entirely obviate this type of lesion, although it gives fewer hæmorrhagic lungs than a blow on the back of the head and therefore is to be preferred to that method. Bleeding by cutting the vessels in the neck has been tried (1) but failed to produce the desired result.

Three pigs out of twenty-three killed in this way had hæmorrhagic lungs. The method of *Experiment I* appears to be successful in avoiding hæmorrhages, but because it interferes with bacteriological examination of the organs it very frequently cannot be employed.

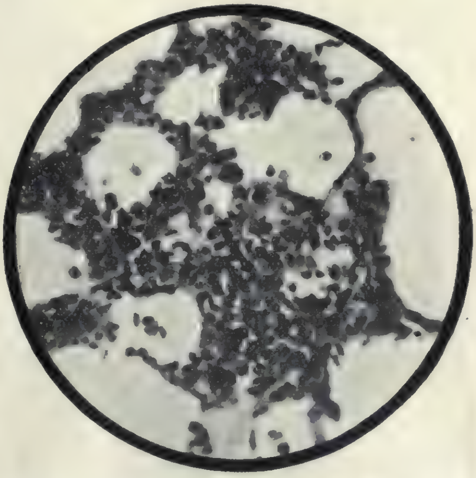


FIG. 5.—Area of hæmorrhage from lung in *Fig.* 4. (× 160.)

Although guinea-pigs were used for the elucidation of this pulmonary lesion, the same type of hæmorrhage was also observed in rabbits and mice which had been inoculated, along with the guinea-pigs, with filtrates of influenzal material during the experiments on influenza. It therefore seems reasonable to believe that the similar lesions in them were caused in the same way as in guinea-pigs and were produced at the time of death. It is necessary to take into account the possibility of these pulmonary hæmorrhages when interpreting *post-mortem* findings in laboratory animals, in the same way as allowance should be made for the proliferative lung lesions. The chief features by which they may be distinguished are the absence of leucocytic reaction in the tissues and the absence of hæmosiderin.

THE PROLIFERATIVE LESION.

The liability of the guinea-pig to spontaneous infections is well known and excellent papers on this subject are available (3) (4). The stock of most laboratories suffers occasionally from epizootics, and in the absence of epidemics the animals may harbour pathogenic bacteria, which do not cause serious infections on account of the immunity which the animals appear to have developed in these circumstances. Theobald Smith (2) points this out in discussing the pneumonia due to *B. bronchisepticus*, and mentions that stock which are carriers of this organism are not suitable subjects for experiments regarding its pathogenicity.

Although the animals used for the influenza experiments appeared to be healthy and the lungs were usually sterile, *B. bronchisepticus* was isolated from the lungs of sixteen guinea-pigs. Five of these showed no gross lesions. The lungs of the remaining eleven (six of which had small hæmorrhages) presented a lesion which in the gross appeared as an irregular-shaped part of one lobe depressed below the level of the normal lung surface. It was a reddish colour, smooth, firm and homogeneous, and extended in a solid, sharply outlined mass from one surface of the lung to the other. Microscopic examination showed that this was a condition of atelectasis. There was no inflammatory reaction. This change was also found in lungs that did not yield any bacteria on culture. Therefore we are not able to associate *B. bronchisepticus* with any constant lung abnormality in this series of guinea-pigs, nor are we able to ascribe to it any ætiological significance deduced from experimental data.

The histological character of the proliferative lesion, which has been described in the former paper (1), showed it to be a slow inflammatory reaction, and it may well be, when one considers the pathogenic properties of the organism, that *B. bronchisepticus* was the ætiological agent, although we cannot go farther than to suggest this possibility.

The microscopical appearance (Fig. 6) of the lesion was as follows: The alveolar walls were thickened, due to proliferation of endothelial cells. Where the change was least the outline of the alveoli could easily be traced, but the air-space of the lung was considerably lessened. In places of greater change single alveoli were completely obliterated by the proliferation; others nearby were only a small fraction of their original size, and the outline of the alveolar walls was lost in the diffuse mass of cells. Where this was most marked the tissue was solid and the air-space entirely obliterated. The cells were rather large and irregular in outline, the nuclei were large, central, oval or round, and stained with moderate intensity. The cell masses included capillaries. Giant cells were occasionally seen. A recent thrombosis of a small branch of a pulmonary artery was associated with this type of lesion.

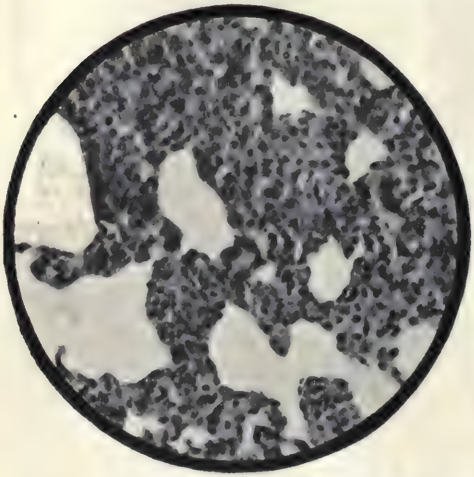


Fig. 6.—Section of the lung of an untreated guinea-pig showing the proliferative change. (× 120.) This section shows proliferation of moderate extent. In other areas the cells were continuous, obliterating all the air-space; the same change also occurred in small patches and was less extensive.

The sixteen strains of *B. bronchisepticus* were identical morphologically and in their cultural appearances, and corresponded to the description of the organism given by McGowan (2), Theobald Smith (3), and Holman.(4) It was a Gram-negative, motile bacillus showing marked pleomorphism. It stained easily and very often unevenly, the ends being more heavily stained than the centre. When first isolated it grew luxuriantly in plain broth or on plain agar in forty-eight hours at 37·5° C., but after several sub-cultures an equal growth appeared after twenty-four hours in the incubator or after forty-eight hours at room temperature.

On agar the colonies varied in size from 1 to 4 mm. in diameter, were discrete, round, moist, greyish-white and translucent. In broth the first cloudiness appeared at the surface. Later the whole became cloudy, a pellicle developed, and a tenacious, white stringy deposit formed in the bottom of the tube.

From agar cultures the organisms tended to be smaller, 0·5 × 1·0 μ, and showed less variation in size and staining. From broth the pleomorphism was marked; filamentous forms and short chains were seen and the organisms were somewhat larger.

Gelatin was not liquefied. Litmus milk was not changed at the end of a week. On potato there was a luxuriant growth, and in from three to seven days it became a deep yellowish-brown colour. Endo's medium was not fermented, likewise neither glucose, lævulose, maltose, mannite, saccharose, galactose, raffinose, salicin, dulcite, dextrin nor inulin.

SUMMARY AND CONCLUSIONS.

A pulmonary lesion in guinea-pigs has been described which was characterised by hæmorrhage into the alveoli and sometimes into the bronchioles. There was no leucocytic reaction in the lung-tissue, and no hæmosiderin was present in the cells of the lung. This lesion is an agonal phenomenon. The same lesion occurred in rabbits and in mice, and is considered in them to be also an agonal phenomenon. Killing by rapid chloroform anæsthesia produced fewer agonal lesions than a blow on the back of the head. A method of killing has been described by which hæmorrhage was not produced.

A second type of lesion has been noted which is essentially a slow proliferation of endothelial cells. It is a spontaneous animal disease whose origin is undetermined.

The hæmorrhagic and proliferative lesion frequently occurred in the same area, but neither was dependent on the other.

B. bronchisepticus was isolated from the lungs of sixteen guinea-pigs, but it was not possible to associate its presence with the proliferative lesion.

REFERENCES.

1. Maitland, H. B., Cowan, Mary L., and Detweiler, H. K.—"The Ætiology of Epidemic Influenza: Experiments in Search of a Filter-passing Virus," *Brit. J. Exper. Pathol.*, 1920, **1**, 263.
2. McGowan, J. P.—"Some Observations on an Epidemic of 'Distemper' among Laboratory Animals," *J. Pathol. and Bacteriol.*, 1911, **15**, 372.
3. Smith, T.—"Some Bacteriological and Environmental Factors in the Pneumonias of Lower Animals, with Special Reference to the Guinea-pig," *J. Med. Res.*, 1913–14, **29**, 291.
4. Holman, W. L.—"Spontaneous Infection in the Guinea-pig," *J. Med. Res.*, 1916–17, **35**, 151.

THE NATURE OF THE EFFECT OF BLOOD-PIGMENT UPON THE GROWTH OF *B. INFLUENZÆ*.*

PAUL FILDES.

From the Bacteriological Laboratory of the London Hospital.

Received for publication November 1st, 1920.

DURING the preparation of peptic digests of blood for the growth of *B. influenzæ* by a technique already described (1), it was found that sometimes a separation of the blood-pigment from the other blood constituents took place. The blood-pigment deposited as a fine dark brown powder, leaving a clear yellow supernatant fluid. If such a mixture was filtered through paper, the precipitate could be gathered, washed with distilled water, redissolved in caustic soda and adjusted to a reaction of P_H 7·6.

The brown precipitate consisted of hæmin, the hydrochloride of hæmatin, and upon being washed and dissolved in soda was converted into alkali hæmatin. The filtrate showed no spectrum of blood-pigment and a "peroxydase" reaction with tincture of guaiacum and ozonic ether only in very low dilutions. Thus it contained extremely small quantities of blood-pigment.

If this alkali hæmatin was added to agar in large or small quantities no growth of *B. influenzæ* was obtained. Similarly the filtrate up to 25 per cent. failed to promote growth, but when small quantities of the hæmatin and filtrate were added together, then a copious growth resulted.

From such an experiment it follows that blood-pigment alone when combined with ordinary nutrient agar is incapable of supporting the growth of *B. influenzæ*, and that a substance present in the non-pigmented fraction of blood is also essential.

By repeating the above experiment with washed red corpuscles instead of with defibrinated blood, it is shown that the two essential factors are still present, apparently in undiminished quantity, and thus it may be assumed that the non-pigmented fraction is derived from the formed elements of the blood.

The object of the present work was to throw light upon the part taken by the pigment in the growth of the bacillus.

It is known that the bloods of different animals behave in different ways towards *B. influenzæ*, and on this account the experiments have been confined to the blood of one animal, namely the sheep. Further, it is known that very

---

* Work carried out under grants from the Medical Research Council.

small quantities of blood-pigment suffice for growth, and therefore in test cultures it is necessary to transfer only minimal quantities of diluted bacteria from a medium poor in pigment.

Six different strains of *B. influenzæ* have been used, and no differences in behaviour have been observed.

THE RELATION OF BLOOD-PIGMENT TO THE GROWTH OF *B. INFLUENZÆ.*

In accordance with the original description of Pfeiffer (2) no growth of *B. influenzæ* has ever been obtained in the absence of blood-pigment.

It will be recalled that the opposite was claimed by Cantani (3), but refuted by Ghon and von Preyss (4), Luerssen (5) and others. The mistake of Cantani was due to his use of the spectroscope as a test for blood-pigment; but it has been shown that quantities of pigment are sufficient which cannot be detected by this test. For instance, the medium of Levinthal is quite colourless and gives no absorption spectrum, but the guaiacum reaction is always positive. The statement is also sometimes met with that *B. influenzæ* can grow without blood-pigment in symbiosis with *Staphylococcus aureus* and other bacteria, but such growths have not been observed in the present work if care is taken to transfer no blood-pigment with the inoculum and if no pigment is present in the medium (guaiac test). It is, however, clear that very small quantities of blood-pigment suffice for growth.

THE QUANTITATIVE RELATION OF BLOOD-PIGMENT TO THE GROWTH OF *B. INFLUENZÆ.*

It has been stated by Davis (6), and his statement has been generally accepted, that 1 in 180,000 of human hæmoglobin is sufficient to allow growth of *B. influenzæ*, but such a high dilution has not been confirmed in the present work with sheep's blood. Davis obtained his results by diluting human blood with distilled water and adding this to agar. He then found the minimum quantity which would allow growth and calculated the amount of hæmoglobin in this quantity. When subcultures were made in series upon the higher dilutions it was found that they were apt to die out.

When repeating these experiments pure alkali hæmatin was used, and added in falling quantities to agar made up with an excess of the colourless filtrate of digested corpuscles. When these tubes were inoculated with *B. influenzæ* growth occurred up to a dilution of 1 in 5 million of hæmatin. The experiments were, however, repeated. A fresh pipette was taken to make each dilution of hæmatin, and the bacteria used for the inoculation were washed once by suspension in saline before being transferred. In these experiments it was found that 1 in 50–63,000 of hæmatin was the limit in four separate series of tests. It may be taken that there is 0·8 per cent. of hæmatin in whole blood, and therefore the necessary dilution of sheep's blood is 1 in 400–500.

It is probable that growth at higher dilutions depends upon error in carrying over blood-pigment from tube to tube or with the bacteria from the original medium, and the fact that Davis's cultures were apt to die out on subcultivation on the higher dilutions supports this view.

THE BLOOD-PIGMENTS WHICH ARE NECESSARY FOR THE GROWTH OF *B. INFLUENZÆ.*

According to Pfeiffer hæmoglobin is essential for the growth of *B. influenzæ*, but it was soon shown that this was not the case. Grassberger (7) and Ghon and Preyss both found that hæmatin would allow growth in symbiosis with *Staphylococcus aureus* but not otherwise. Similarly Voges showed that heated blood containing no hæmoglobin but hæmatin was a good medium, but both Grassberger and Ghon and Preyss pointed out that the formation of hæmatin could not be the factor, because hæmatin, whether heated or not, did not support growth. In a later paper, however, Ghon and Preyss (1904) (4) found that hæmatin would allow growth without symbiosis if a little sterile milk was smeared upon the surface of the medium.

Looking at these original experiments with later knowledge, it is clear that the failure to obtain growths with hæmatin was due to the absence of the essential body which is contained in the colourless fraction of blood-corpuscles. This factor, it may be presumed, is supplied in a slight degree by milk or by the action of *Staphylococcus aureus.*

Nearly all early writers tested the effect of other blood-pigments without success.

In the present work it was not possible to obtain pure hæmoglobin from the sheep, and therefore the experiments were carried out with admittedly impure samples prepared as follows: 100 c.c. of washed deposited sheep's corpuscles were placed in a stoppered bottle with an equal volume of salt solution and 40 c.c. of ether. The mixture was thoroughly shaken, allowed to stand on ice overnight, and the hæmoglobin was then removed from the supernatant stroma. By warming and shaking most of the ether was removed, and the solution was then filtered through a Berkefeld filter.

Pure specimens of sheep's hæmatin were prepared by two methods—by alcoholic extraction of a dry mixture of anhydrous potassium carbonate and defibrinated blood, and also by the peptic digestion of sheep's corpuscles and the separation of hæmin. In each case the pure dry product was dissolved in water or dilute soda solution with subsequent neutralisation, in a strength of 2 per cent.

A specimen of impure hæmatoporphyrin was obtained from Dr. Laidlaw. Upon analysis by Mr. Candy this was found to contain small quantities of iron—whether in the form of hæmatin or not was not determined.

Attempts to use hæmochromogen in a culture of *B. influenzæ* were abortive. Firstly, it was not possible to reduce hæmatin with the feeble reducers and at the slight degree of alkalinity permissible in a culture medium. Secondly, as will appear later, the presence of any reducing substance, even so feeble a substance as glucose, inhibits or seriously interferes with the growth of *B. influenzæ.*

All blood-pigment solutions were sterilised and preserved by means of chloroform according to a technique previously described. (8)

When testing these preparations they were mixed with ordinary nutrient agar in such quantities as would bring the concentration of pigment in the medium to the equivalent of 1 in 80 of whole blood. It is not necessary to remove the chloroform except when a liquid medium is being used.

The results of the tests were as follows :

Hæmoglobin.—The results with hæmoglobin alone in agar were somewhat variable. Sometimes no growth was observed, but at others it was possible to detect a number of extremely fine colonies in twenty-four hours. When mixed with the colourless filtrate the growth was more constant and pronounced, but even then not invariable and when present always small.

It will be understood that the determination of the presence or absence of growth is a relatively gross matter. This is particularly the case with *B. influenzæ* when grown upon blood-agar made from human or some other bloods. In twenty-four hours the growth is often so excessively fine as almost to escape observation, especially when the blood has been laked before mixing with the agar. In the present paper by growth is meant not the type of growth which is obtained on Pfeiffer's blood-smeared agar, but the copious growth, like that of a young typhoid culture, which is obtained upon heated blood, peptic blood agar (1), or good specimens of Levinthal's medium. On the other hand, by no growth is meant that no colonies could be seen on the medium with a hand-lens after forty-eight hours.

When hæmoglobin is mixed with a medium and incubated the pigment rapidly changes to methæmoglobin, and therefore the use of the term "hæmoglobin" is inaccurate. There is some reason to suppose that this change is responsible for some of the variation observed in the growth obtained. In old specimens of hæmoglobin, largely converted into methæmoglobin, the growth tends to be more obvious than when the pigment is fresh, and it is probable that were it possible to maintain the pigment as hæmoglobin no growth would occur.

Hæmatin.—As already indicated, hæmatin mixed with agar never grew *B. influenzæ*, but copious growths were obtained when the colourless fraction of a peptic digest was added.

Hæmatoporphyrin.—This substance failed to grow the bacillus either alone or when mixed with the colourless fraction.

From these experiments it was concluded that an iron-containing pigment was necessary for the growth of *B. influenzæ*, and that the protein-containing pigment was very much less effective than that free from protein.

The necessity for iron was recognised by Pfeiffer and later workers, and they had attempted to reintroduce it in the form of various organic and inorganic preparations without success. I have tested the effect of ferric ammonium citrate, dialysed iron, colloidal iron, metallic iron, and inorganic ferric and ferrous salts both alone and with the colourless filtrate or with the filtrate and hæmatoporphyrin without obtaining growth of *B. influenzæ*, but such results need merely indicate that the experimentally-introduced iron is not in the same state or under the same conditions as the iron of hæmatin.

THE OXYGEN REQUIREMENTS OF *B. INFLUENZÆ*.

The fact that blood-pigments are associated with the function of oxygen-carrying, and that iron salts are believed to act as catalysts to accelerate the oxidation of materials in the life of cells, led to experiments being carried out upon the relation of *B. influenzæ* to oxygen.

It is known that *B. influenzæ* is a strong aërobe, according to some (9) an obligate aërobe, and it is stated not to grow under anaërobic conditions. Further, it is known that good growths in liquid media are only to be obtained when a large surface of the medium is exposed to the air. The growth takes place almost entirely in the superficial layers of the culture, and therefore the relation of the aërated surface to the bulk of the medium has a great bearing upon the thickness of the growth when the culture is shaken up and dispersed. A liquid medium, 2 or 3 mm. deep, in a flask will grow very much more copiously than the same bulk of medium in a tube. A copious growth is thus dependent upon a rich supply of oxygen.

If, however, slopes of medium containing peptic digest of blood-corpuscles are inoculated with *B. influenzæ* and incubated under anaërobic conditions (McIntosh and Fildes' anaërobic jar (10)), a growth of minute colonies will still be found in twenty-four hours—more distinct, in fact, than an aërobic growth on Pfeiffer's blood-smeared agar. If, after two or three days' incubation, this culture is subcultured and this subculture incubated aërobically no growth will occur, and it is thus clear that under such anaërobic conditions *B. influenzæ* will grow normally up to a certain point and will then die out. From this experiment it follows either that the anaërobic apparatus in use had not extracted all of the atmospheric oxygen and that a remnant had been left to allow this growth, or that there is an oxygen store in the medium which is not capable of being extracted by a simple process of diffusion.

The method of oxygen absorption with spongy palladium in an atmosphere of hydrogen is well known to be suitable for the surface growth of strict anaërobes, and therefore it is improbable that sufficient atmospheric oxygen would be left to allow such a considerable growth of a strong aërobe. The following experiment indicates that the oxygen is not obtained from the atmosphere, but from the medium :

Two small plates containing a very thin layer of agar medium *plus* the peptic digest were placed in a McIntosh and Fildes' anaërobic jar. A magnetic apparatus was also included, by which a small drop of a suspension of *B. influenzæ* in broth could be dropped upon the plate from a pipette without opening the jar. A drop of this suspension having been placed upon plate 1, the apparatus was shut, the oxygen absorbed and the jar incubated for two days. A drop of the suspension was then placed upon plate 2 and after a further incubation of two days the jar was opened. On each plate was an equal, very feeble growth of *B. influenzæ*. These were subcultured, and the bacteria on plate 1 were shown to be dead but those on plate 2 living. Thus, whatever oxygen the atmosphere of the jar contained at the beginning of the experiment, there was not enough left to support growth continuously on plate 1; nevertheless, plate 2 grew equally well under these conditions and therefore must have derived its oxygen from the medium, although the bulk of this was small and the area for diffusion great.

If experiments are made under conditions which would tend to deprive the medium still more of dissolved or loosely combined oxygen, namely, if the jar is repeatedly exhausted and flooded with hydrogen, then the growth of *B. influenzæ* becomes still less and may even become inappreciable.

The dependence of *B. influenzæ* upon a store of oxygen in the medium is also shown by its behaviour in shake cultures. Deep agar tubes containing the peptic digest were placed in boiling water until similar tubes containing methylene-blue in addition were completely reduced. The deep tubes were then rapidly cooled, inoculated with *B. influenzæ* upon a fine glass rod and set in cold water. A fine glass rod was found to be less liable to introduce air than a pipette. The zone of anaërobic conditions in these inoculated tubes could be judged by comparison with the methylene-blue tubes similarly treated, but with an uninfected rod.

As would be expected with an aërobic organism, growth occurred in the first twenty-four hours upon and just under the surface, but later growths appeared below the level of aërobic conditions and extended right to the bottom of the tube, where there was a complete absence of active oxygen. In other experiments it was shown that growth also took place actually in the presence of reduced methylene-blue, but the results were less constant.

If glucose is added to these tubes a result is obtained comparable to the pumping and flushing of the jar. The store of oxygen becomes less available and the growth is less marked and may be absent.

It is probable that the inconstant results of growth in the presence of reduced methylene-line blue is due to the blue acting similarly as a reducing agent. It may therefore be assumed that there is in the medium a store of oxygen upon which the bacteria may draw until it is used up, and that then they die. Under aërobic conditions the store is replenished from the atmosphere. When a reducing substance such as glucose is present in sufficient quantity, the oxygen store may be largely used up and the bacteria deprived of it. The growth under conditions which would remove merely dissolved oxygen, namely, pumping in the case of a thin layer of medium or boiling in the case of a shake culture, and the fact that growth will take place actually in the presence of colourless methylene-blue, indicate that the oxygen store is not merely dissolved in the medium, but is inactive in a state of loose combination with some substance in it.

It may be supposed that the medium contains an easily oxidisable and reducible substance—a "peroxide"—which is the direct source of oxygen to the influenza bacillus, and is immediately reoxidised in the presence of air. Such "peroxides" are familiar in connection with theories relating to the biology of cells in the body, and the activity of these substances is thought to be accelerated by the catalytic action of iron. Thus, it appeared possible that the iron of hæmatin might act towards this hypothetical "peroxide" or store of fixed oxygen in the medium as a catalyst, in order to accelerate the reduction of the peroxide by the bacterium.

THE FUNCTION OF THE HÆMATIN.

Pfeiffer himself thought that hæmoglobin might act as an oxygen carrier to the bacteria, but discarded the theory on the ground that carboxyhæmoglobin, which could not be an oxygen carrier, could nevertheless permit the growth of *B. influenzæ.* Pfeiffer was looking upon the oxygen function of the blood-pigment as similar to the function exerted in nature, but experiments with carboxyhæmoglobin and the fact also that hæmatin is more effective make this view improbable.

From the present work the function of the hæmatin might be considered to be two-fold: first, it might act as an iron salt as a catalyst, and secondly, it might act as a "peroxide," being a reducible substance easily re-oxidised in air.

There are some observations which oppose this second possible function and others which favour it. Firstly, it might be supposed that if hæmatin functioned as a peroxide, a shake culture of *B. influenzæ*, after several days' growth should reduce hæmatin in the deeper parts of the tube, and show the spectrum of reduced hæmatin, namely of hæmochromogen. This spectrum is not, however, observed; but, in fact, it cannot be observed, since it requires a greater concentration of alkali than is permissible in a culture of *B. influenzæ*. Thus, if a drop of N/1 NaOH is placed upon the surface of a deep agar culture of *B. influenzæ*, the spectrum of hæmochromogen spreads down into the depth of the tube with the alkali, and if the tube be cut at the spreading edge and the surface tested with thymolphthalein the reaction will be found to be over P_H 9·0. At this reaction *B. influenzæ* will not grow. This effect of alkali is not, however, dependent upon the growth of *B. influenzæ*, because the same result may be observed in an uninoculated tube, in which the mere reducing power of the medium has introduced anaërobic conditions. On the other hand, there is some reason to suppose that hæmatin may be reduced without the production of hæmochromogen. Bertin-Sans and Moitessier ([11]), for instance, describe a reduced hæmatin which does not give the spectrum of hæmochromogen, and further, the same authors note the existence of an oxidised product of hæmochromogen which differs from hæmatin in giving the bands of oxyhæmoglobin. In this connection it is of interest to remark that the bands of oxyhæmoglobin appear sometimes, under conditions undetermined, when the soda is added to the culture, as described above. Thus the absence of hæmochromogen bands from the culture does not necessarily imply that the hæmatin has not been reduced, since it is demonstrated that other reduced compounds than hæmochromogen exist. If, however, hæmatin functioned as a peroxide, the growth of *B. influenzæ* under anaërobic conditions should be proportional to the amount of hæmatin in the medium, but in practice this is not the case. If sufficient hæmatin is present to allow a meagre growth, this cannot be increased by adding much larger quantities.

The evidence is therefore, on the whole, against the probability that hæmatin functions as a peroxide in the medium.

The view of a catalytic effect of the hæmatin is supported by a certain amount of indirect evidence. It will be remembered that a catalytic action of blood-pigment is familiar in the misnamed "peroxidase reaction" of blood with tincture of guaiacum and ozonic ether. Ozonic ether, a peroxide, is alone practically incapable of oxidising the guaiaconic acid of the tincture, but upon the addition of a trace of blood the reaction is enormously accelerated, and the oxidisation takes place immediately with the formation of a blue colour. As ordinarily carried out with blood this reaction depends upon the added peroxide, but it is found that hæmatin is capable of oxidising the guaiacum alone without added peroxide, apparently by utilising dissolved oxygen or oxidised components of the resin.

These tests were carried out under conditions which simulated those under which *B. influenzæ* grows. The various pigment solutions were diluted with nutrient broth to a dilution representing 1 in 32 of blood. The reaction was then adjusted exactly to P_H 7·2 (cresol-red). 5 c.c. of these dilutions were added to 5 c.c. of tincture of guaiacum diluted with distilled water, and the whole warmed for a minute or two and then left at room temperature.

Under these conditions laked blood, laked blood-corpuscles or fresh oxyhæmoglobin solutions gave no blue colour. Preserved hæmoglobin solutions containing methæmoglobin (these usually reacted acid to methyl-red) gave a slight blue colour which tended to disappear on standing. Peptic digest of blood gave rapidly a strong blue colour. Hæmatin gave a slowly developing but strong blue colour, and hæmatoporphyrin (containing, as mentioned, iron) a varying intensity of blue.

Thus there is a marked parallelism between this reaction and the growth of *B. influenzæ*. In the presence of oxyhæmoglobin little or no growth occurs and this reaction is negative. With old hæmoglobin preparations usually a feeble growth occurs and a feeble guaiacum reaction. The peptic digest or hæmatin permits a profuse growth and gives a strong reaction. Hæmatoporphyrin gives no growth, but the reaction is no doubt due to remnants of iron. Pure hæmatoporphyrin is said to give no guaiacum reaction even with ozonic ether.

The comparison may be carried even further in a striking manner. If to a medium containing peptic digest of blood and capable of producing a copious growth of *B. influenzæ* fresh laked blood, laked corpuscles or fresh hæmoglobin solutions are added, the growth is almost entirely or entirely inhibited. Similarly, the peptic digest *plus* fresh blood, etc., will give no guaiac reaction—in fact, a strong blue colour which has developed with peptic digest alone may be discharged by the addition of fresh hæmoglobin. Again, pigeon's blood is the only blood which has been found in the present work to give a profuse growth of *B. influenzæ* in its fresh state, and it is the only one which has been found to give a strong guaiac reaction.

From these experiments, therefore, although the evidence is indirect, it appears that the relation of blood-pigment to the growth of *B. influenzæ* is the same as the relation of the pigment to the oxidisation of guaiaconic acid; in fact, that the pigment acts as a catalyst to accelerate the transfer of oxygen from a peroxide in the medium to the bacillus.

THE NATURE OF THE DIFFERENCE BETWEEN HÆMATIN AND HÆMOGLOBIN AS MEDIA.

The present experiments throw some light upon the well-known phenomenon of the feeble or absent growth of *B. influenzæ* on "unchanged" blood, and the copious growth on blood "changed" by heat, digestion, or other methods. In the changed bloods the pigment is in the form of hæmatin and in the unchanged in the form of mixtures of hæmoglobin and methæmoglobin. In the former case the pigment has a marked catalytic action, but in the latter, except in the case of pigeon's blood, there is little or no catalytic effect demonstrable by the technique in use. This absence of effect may be due to some character of the structure of hæmoglobin rendering it feebly catalytic, or it may be due to an inhibitory action. There is no reason to suppose that the catalytic action of hæmoglobin is inherently feeble, since it rapidly causes an oxidisation of guaiac upon the addition of ozonic ether. There is also direct evidence of an active reducing effect upon guaiac rendered blue by the action of hæmatin, or even guaiac rendered blue by the action of milk.

It is possible that, owing to its affinity for oxygen, hæmoglobin may take up any oxygen which becomes available by its catalytic action and thus deviate it from the bacillus, in the same way as glucose prevents growth and prevents the development of the guaiac reaction. This explanation might account for the greater growth which is obtained with old specimens, in which much of the hæmoglobin is changed to methæmoglobin before even the culture is incubated. On the other hand, experiments with carboxyhæmoglobin are not sharply confirmative. If a fresh hæmoglobin solution is placed in a thin layer in a flask and exhausted with the pump, pure carbon monoxide may be admitted and shaken with the pigment. The carboxyhæmoglobin so obtained may be incorporated in a medium and tested. The growth with carboxyhæmoglobin is practically the same as with hæmoglobin and so is the catalytic action, and yet this pigment has no affinity for oxygen. Spectroscopic examination, however, shows that carboxyhæmoglobin also is not stable in a medium. Indeed, it would be expected that in the course of incubation some would be reconverted on the surface and thus an inhibitory factor would be again introduced.

On the whole it appears probable that the feebleness of the growth upon an unchanged blood medium is due, at any rate in part, to the inhibitory action of hæmoglobin, this inhibition resulting from the intercepting of oxygen liberated from a peroxide by the catalytic action of iron in the pigment. Any process, such as slight heating or digestion or the addition of acid or alkali, which destroys the oxygen avidity of the pigment, at once allows the full catalytic action to operate and a copious growth occurs. According to this view such feeble growth as does occur upon unchanged blood media is due to the inevitable change to methæmoglobin and therefore a reduction in the inhibitory factor.

CONCLUSIONS.

(1) Growth of *B. influenzæ* has not been observed to occur in the total absence of blood-pigment.

(2) The quantity of blood-pigment necessary is small, but larger than has been stated.

(3) Probably oxyhæmoglobin and carboxyhæmoglobin are incapable of allowing growth, the feeble multiplication in these pigments being due to a spontaneous change to methæmoglobin. Hæmatoporphyrin also fails to permit growth, but hæmatin allows a copious growth.

(4) *B. influenzæ* on suitable media grows fairly well under anaërobic conditions, but rapidly dies out. The necessary oxygen is obtained from an oxygen store in the medium. This store is not dissolved active oxygen, but is inactive in a state of loose combination with a constituent of the medium. Under aërobic conditions the combined oxygen is used up by the bacillus and immediately re-formed from the atmosphere.

(5) The combined oxygen in the medium is activated for the bacillus by the catalytic action of iron in the pigment.

(6) The feebleness of the growth on unchanged blood compared with that upon changed blood is due to a deviation of the oxygen, activated by the iron of the pigment, from the bacillus through the oxygen affinity of the unchanged pigment.

REFERENCES.

1. Fildes, P.—"A New Medium for the Growth of *B. influenzæ*," *Brit. J. Exper. Pathol.*, 1920, **1**, 129.
2. Pfeiffer, R.—"Die Ætiologie der Influenza," *Zeitschr. f. Hyg. u. Infektionskrankh.*, 1893, **13**, 357.
3. Cantani, A., Jun.—"Ueber das Wachsthum der Influenzabacillen auf hämoglobinfreien Nährböden," *ibid.*, 1901, **36**, 29.
4. Ghon, A., and v. Preyss, W.—"Studien zur Biologie der Influenzabacillus," *Centralbl. f. Bakteriol.*, Orig., 1902, **32**, 90; 1904, **35**, 531.
5. Luerssen, A.—"Beiträge zur Biologie der Influenzabacillus," *ibid.*, Orig., 1904, **35**, 434.
6. Davis, D. J.—"Hemophilic Bacilli: Their Morphology and Relation to Respiratory Pigment," *J. Infect. Dis.*, 1907, **4**, 73.
7. Grassberger, R.—"Zur Frage der Scheinfädenbildung in Influenzakulturen," *Centralbl. f. Bakteriol.*, 1898, **23**, 353.
8. Fildes, P.—"The Technique of the Preparation of Culture Media Containing Albuminous Fluids," *Lancet*, 1917, **1**, 492.
9. Gotschlich, E.—"Allgemeine Morphologie und Biologie der pathogenen Mikroorganismen," in Kolle and Wassermann's *Handbuch*, Jena, 1912, **1**, 91.
10. McIntosh, J., and Fildes, P.—"A New Apparatus for the Cultivation of Anaërobic Micro-organisms," *Lancet*, 1916, **1**, 768. "An Improved Form of McIntosh and Fildes' Anaërobic Apparatus," *Brit. J. Exper. Pathol.* (shortly).
11. Bertin-Sans, H., and Moitessier, J.—"Oxyhématine, hématine réduite et hémochromogène," *Compt. rend. Acad. d. Sci.*, 1893, **116**, 401.
12. Olsen, O.—"Ueber die Bedeutung des Blutes für das Wachstum des Pfeifferschen Influenzabacillus," *Centralbl. f. Bakteriol.*, Orig., 1920, **85**, 12.

NOTE.

In a current number of the *Centralbl. f. Bakteriol.*, Olsen (^{12}) also has arrived at the conclusion that the action of the blood-pigment is catalytic from the fact that iron-containing pigments grow the bacillus and give a positive guaiacum reaction with peroxide of hydrogen, while pigments which do not contain iron do neither. He does not account for, nor discuss, the difference in growth observed in the presence of hæmoglobin and hæmatin.

HÆMAGGLUTINATION AND ITS MEDICO-LEGAL BEARING, WITH OBSERVATIONS UPON THE THEORY OF ISOAGGLUTININS.

H. SCHÜTZE, M.D.

From the Bacteriological Department, Lister Institute, London.

Received for publication November 14th, 1920.

THE precipitin test has long been accepted as a means of differentiating one species of blood from another. Should the specimen be a human one, it might, however, be of the greatest medico-legal importance to carry the investigation one step further, and ascertain the iso-agglutination group* to which the specimen belongs. The necessity of investigating the possibility of grouping according to Moss (¹) blood which is already dry arose for the author in connection with a case where material, alleged to be blood supernaturally pouring from a holy picture, was found to react positively with anti-human precipitin serum, and it was desirable to exclude or include certain persons as possible sources of supply.

No work appears to have been done to establish the possibility of grouping blood that has dried, or to find out for how long after desiccation such grouping might be practicable, but that a serum reconstructed from freshly dried blood still contains hæmagglutinins is seen from the following experiment:

Experiment I.—One c.c. of a Group II blood dried overnight in a Petri dish was reconstructed the following morning by rubbing up the material in 1 c.c. of distilled water with pestle and mortar and centrifuging to get rid of the undissolved residue. The hæmoglobin-stained supernatant was then used, alongside of serum obtained from the same individual, for the agglutination of red cells of known type.

Serum.	Red cell.	Serum dilutions.						
		$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$
Group II (Fresh) . .	Group I . .	‡	‡	‡	‡	‡	+	—
	Group III .	‡	‡	‡	‡	‡	+	—
Group II (Reconstructed after 1 day) . .	Group I . .	‡	‡	‡	‡	+	—	—
	Group III .	‡	‡	‡	‡	+	—	—

(‡ = macroscopic clumping; + = microscopic clumping.)

* The reactions of the Moss groups are as follows:

Serum.	I.	II.	III.	IV.
Cell I . .	—	+	+	+
Cell II . .	—	—	+	+
Cell III . .	—	+	—	+
Cell IV . .	—	—	—	—

(+ = agglutination of red cell by serum.)

In these tests the serum dilutions are carried out with physiological saline; the red cell suspension is a saline one containing 1·5 per cent. sodium citrate, and of such a density that a tiny platinum loopful added to a capillary drop (about 0·01 c.c.) of serum gives a concentration found convenient for the reading of results. This method was found to be sufficiently accurate to ensure quantitative reliability. The glass slide holding such drops is placed in a moist chamber at room temperature. Reading usually followed after one hour.

Confronted, as one would be in practice, with an unknown quantity of blood either in dry scale form or soaked into some material, one would have to arrive at quantitative measurements by working from a weighed amount of the dry blood, and reconstructing with sufficient distilled water to reproduce the volume of blood the dry sample represented.

Should such a reconstructed serum prove to contain no agglutinins, one would still have to decide whether the blood belonged to Group I, which possesses none, or whether its agglutinins had disappeared since the shedding of the blood. This decision might possibly be arrived at by establishing the character of the antigen presumably still existent in the undissolved residue spun down after reconstruction, by absorption of a Group IV serum with the residue and seeing what, if any, agglutinins were removed.

As a preliminary, absorption tests were carried out with fresh cells of the four groups (see Experiment II). Incidentally, also, it was intended to see which of the theories, that of Moss (1), or that of Landsteiner (2) as amplified by Jansky (3) and later by v. Dungern and Hirschfeld (4), best fits the facts.

Moss, as is seen in the following table, originally put forward two explanations, the first of which he discarded on the strength of an absorption experiment, viz. the saturation of a Group IV serum with Group I cells, when he found that no agglutinins for either Group II or Group III cells remained, which result was incompatible with theory No. 1. Apparently he did not proceed to further cross-absorptions, so that Happ (5), in a recent paper also from the Johns Hopkins Hospital, still regards the second hypothesis as a possibility.

Moss's blood groups.	Moss's theory 1.		Moss's theory 2.		v. Dungern and Hirschfeld's theory.	
	Cell.	Serum.	Cell.	Serum.	Cell.	Serum.
I	A	—	A+B+C	—	A+B	—
II	B	a+c	B+C	a	A	b
III	C	a+b	A+C	b	B	a
IV	—	a+b+c	—	c	—	a+b

(Capital letters = antigens; small letters = agglutinins.)

If further absorptions had been performed, it would at once have been evident that Moss's second theory also was disproved.

Von Dungern and Hirschfeld's theory is simply an extension of that of Landsteiner to include Group I. This group, owing to its rarity, had been overlooked, and only the three other ones described. These workers assume the existence of but two antigens in the red cells of mankind—an antigen A, present in the cells of Group II individuals, and an antigen B in the cells of those of Group III. Both antigens are found together in Group I cells, while those of Group IV contain neither; these Group IV cells are devoid of agglutinable antigen. The agglutinin content of the serum shows a curious reciprocity. Where there is a certain antigen in the cell, there the corresponding agglutinin is absent from the serum.

Group II has antigen A in the cell and agglutinin b in the serum, while Group III has B in the cell and a in the serum. As Group I cells contain both A and B, there is no agglutinin of either sort in the serum. Group IV, which has no antigen in its cells, has both agglutinins in its serum.

Von Dungern and Hirschfeld bring forward no absorptive evidence in proof of their theory, and are apparently convinced of its correctness by the simplicity with which it meets the facts of agglutination.

Experiment II.—Absorption in Experiment II was performed by mixing 0·4 c.c. serum with 0·4 c.c. of the centrifuged deposit of the washed red cells in question, the mixture standing at room temperature for 2 hours with intermittent shaking.

Serum IV.	Unabsorbed.								Absorbed with Cell I.							
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$
Cell I . .	++	++	++	++	++	++	++	—	++	+	—	—	—	—	—	—
Cell II . .	++	++	++	++	++	++	++	—	++	—	—	—	—	—	—	—
Cell III . .	++	++	++	++	++	+	—	—	++	+	—	—	—	—	—	—

Serum IV.	Absorbed with Cell II.								Absorbed with Cell III.							
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$
Cell I . .	++	++	++	++	+	—	—	—	++	++	++	++	++	++	+	—
Cell II . .	++	—	—	—	—	—	—	—	++	++	++	++	++	++	+	—
Cell III . .	++	++	++	++	+	—	—	—	++	—	—	—	—	—	—	—

Serum IV.	Absorbed with Cell IV.							
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$
Cell I . .	++	++	++	++	++	++	+	—
Cell II . .	++	++	++	++	++	++	—	—
Cell III . .	++	++	++	++	+	—	—	—

From Experiment II it will be seen that while it is true that Moss' first theory is disproved by the fact that Group I cells remove from a Group IV serum the agglutinins for both Groups II and III as well as Group I cells, it is also true that his second theory cannot meet the facts of the experiment. Absorption of a Group IV serum with Group II or III cells does not remove the agglutinins for Group I cells as it would according to Moss, but, on the contrary, leaves them practically intact. On the other hand, it will be seen that the assumption that there are two substances, "A" and "B," in human red cells, with corresponding agglutinins, "a" and "b," in the serum, disposed in the manner indicated by v. Dungern and Hirschfeld, is sufficient to explain not only the agglutination phenomena but those of absorption as well.

Experiment III.—To test the possibility of grouping the residue from a reconstructed blood, the centrifuged deposit left over from Experiment I was washed in saline, and re-suspended in 0·5 c.c. of the same fluid. Of this suspension 0·3 c.c. was added to 0·3 c.c. of Group IV (D)[*] serum, and the mixture allowed to stand for 2 hours at room temperature, being shaken from time to time. As the Group II cells (R) were known from previous experience to be less agglutinable than certain others also belonging to Group II, absorption of a second quantity of Group IV (D) serum was similarly carried out with the undissolved residue from the more agglutinable Group II cells (F), and agglutination demonstrated with both homologous and heterologous examples of well and less well agglutinating Group II cells. Corresponding absorptions, but with fresh washed cells (as in Experiment II) from the same persons, were undertaken at the same time.

Serum IV (D).	Unabsorbed.						Absorbed with Cell II (F).					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell I (J) . .	++	++	++	++	++	—	++	++	++	+	—	—
Cell II (S) . .	++	++	++	++	++	—	++	—	—	—	—	—
Cell II (F) . .	++	++	++	++	++	—	++	—	—	—	—	—
Cell II (A) . .	++	++	++	++	+	—	—	—	—	—	—	—
Cell II (R) . .	++	++	++	—	—	—	—	—	—	—	—	—
Cell III (Z) . .	++	++	++	—	—	—	++	++	++	—	—	—

Serum IV (D).	Absorbed with Residue II (F).						Absorbed with Cell II (R).					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell I (J) . .	++	++	++	+	—	—	++	++	++	++	+	—
Cell II (S) . .	++	—	—	—	—	—	++	++	++	++	+	—
Cell II (F) . .	++	—	—	—	—	—	++	++	++	++	+	—
Cell II (A) . .	—	—	—	—	—	—	++	++	—	—	—	—
Cell II (R) . .	—	—	—	—	—	—	++	—	—	—	—	—
Cell III (Z) . .	++	++	++	+	—	—	++	++	++	—	—	—

Serum IV (D).	Absorbed with Residue II (R).					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell I (J) . .	++	++	++	++	++	+
Cell II (S) . .	++	++	++	++	++	—
Cell II (F) . .	++	++	++	++	++	+
Cell II (A) . .	++	++	+	—	—	—
Cell II (R) . .	++	—	—	—	—	—
Cell III (Z) . .	++	++	++	—	—	—

It will be seen that absorption is as successful with blood residues as it is with fresh cells, and badly agglutinating cells are, as might be expected, also badly absorptive ones; but although saturation with such badly agglutinating Group II cells does not alter the titre of the serum for the better agglutinating Group II cells (S) and (F), the titre as registered by the more or less poorly agglutinating Group II cells (A) and (R) is definitely affected.

---

[*] Bracketed initials indicate the individual from whom the material was obtained.

The recognition of the existence of poorly agglutinating Group II cells—(the cells of 5 individuals out of 13 of Group II have been found to possess this characteristic in more or less marked degree; on the other hand, those of 9 persons of Group III which have been tested were all equally agglutinable)—is of importance for two reasons. When a serum whose titre may from one cause or another have suffered is being grouped, it is obviously advisable to choose a cell with maximal agglutinating powers; while if cell-suspension or residue of unknown nature is being grouped by the absorption method, it is, on the contrary, better to employ a weak cell for agglutination, as being a better general indicator of an eventual decrease in the agglutinin content of the serum.

The possibility of grouping dried blood by agglutinating with its reconstructed serum and absorbing with the cell residue has been demonstrated, but it still remains to be seen how long the antigens and antibodies concerned remain diagnosable; this must no doubt depend on the original titre and the influences to which the dried blood has been subjected.

The present observations extend over a number of months, and grouping has been possible in every case.

In Experiment IV a low-titred blood is examined after 41 days' exposure to sunlight, the specimen being dried on cloth.

In Experiments V and VI high- and low-titred sera are examined 5 months after drying; in each case serum taken at the same bleeding and preserved bottled, in the cold room, is examined for an indication of the keeping qualities of hæmagglutinin sera.

Experiment IV.—Blood of Group III (St) was taken; 0·5 c.c. was dried on a piece of clean linen and exposed to the air on a shelf near a window, where much indirect and some direct sunlight fell upon it, and reconstructed as usual by rubbing and squeezing in a mortar, *but with* 1 *c.c.* of distilled water, after a period of 41 days.

Agglutination and absorption tests resulted as follows:

Serum III (St).	Fresh.				Reconstructed after 41 days on cloth exposed to sunlight.		
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Cell II (S)	‡	‡	‡	—	‡	‡	—

Serum IV (M).	Unabsorbed.					Absorbed with Residue III (St), reconstructed after 41 days.				
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
Cell I (J)	‡	‡	‡	+	—	‡	‡	—	—	—
Cell II (S)	‡	‡	‡	+	—	‡	‡	+	—	—
Cell III (Sa)	‡	‡	+	+	—	—	—	—	—	—

The titre has fallen slightly but the insoluble residue still shows marked avidity for its corresponding Group III cell agglutinins.

Experiment V.—In Experiment V blood of Group II (R) was taken; this individual constantly yields a high-titred serum with cells of poor agglutinability. One c.c. dried in a Petri dish was reconstructed and titred the following day; two similar specimens, one kept in a cupboard sheltered from light and the other exposed to it at a window, and both at room temperature, were reconstructed after 5 months, and examined together with a sample of the same bleeding kept, *as serum*, bottled and in the cold room.

The following titres were obtained:

Blood.	Particulars.	Titre against Cell I (J) and Cell III (Z).
II (R) undried . .	Serum at time of bleeding	$\frac{1}{64}$
,, dried . .	Reconstructed after 1 day	$\frac{1}{32}$
,, ,, . .	Reconstructed after 5 months, sheltered from light	$\frac{1}{8}$
,, ,, . .	Reconstructed after 5 months, exposed to light	$\frac{1}{4}$
,, undried . .	Serum after 5 months in the cold room	$\frac{1}{32}$

The five-months-old dried blood still retains sufficient agglutinin to make the grouping of it an easy matter; even the specimen freely exposed to sunlight, though of a lower titre than the one preserved in the dark, yields a serum capable of agglutinating Group I and III cells in a dilution of 1 in 4. The serum preserved for diagnosis purposes in the cold room has lost during the same period but little of its agglutinating power.

Experiment VI.—In Experiment VI, blood Group II (F) was used; the donor of this has, in contra-distinction to (R) of the previous experiment, always been found to have a low-titred serum with easily agglutinable red cells, but this inverse ratio of agglutinability of cell to titre of serum does not appear to hold good in all cases. One c.c. of Group II (F) was dried and reconstructed after one day; a further c.c. was dried, exposed to sunlight for 5 months and then examined; in both cases the absorption power of the insoluble residue was tested. The keeping qualities of a low-titred serum at 0° C. was also observed.

Blood.	Particulars.	Titre against Cell I (J) and Cell III (Z).
II (F) undried . .	Serum at time of bleeding	$\frac{1}{8}$
,, dried . .	Reconstructed after 1 day	$\frac{1}{4}$
,, ,, . .	Reconstructed after 5 months, exposed to light	$\frac{1}{1}$
,, undried . .	Serum after 5 months in the cold room	$\frac{1}{4}$

Serum IV (D).	Unabsorbed.						Absorbed with Residue II (F), reconstructed after 1 day.					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell II (S) . .	++	++	++	++	++	—	++	—	—	—	—	—
Cell II (R) . .	++	++	++	—	—	—	—	—	—	—	—	—
Cell III (Z) . .	++	++	++	—	—	—	++	++	++	+	—	—

Serum IV (D).	Unabsorbed.						Absorbed with Residue II (F), reconstructed after 5 months, exposed to light.					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell II (S) . .	++	++	++	++	++	++	++	++	++	++	—	—
Cell II (R) . .	++	++	++	++	—	—	—	—	—	—	—	—
Cell III (Z) . .	++	++	++	++	—	—	++	++	++	++	—	—

The low-titred blood Group II (F) after 5 months' exposure to light in a desiccated condition has just sufficient agglutinating power left to indicate the group to which it belongs when tested in undiluted strength.

The complementary absorption test, however, establishes the group with more definiteness, although the specimen shows deterioration in this respect, too. Here again it is seen that less readily agglutinated cells, such as Group II (R), serve best as an indication of absorption. While the titre of the Group IV (D) serum is only somewhat lowered for the easily agglutinable cell Group II (S), that for cell Group II (R) is more markedly affected ; the alien groups cells III (Z) agglutinate to the same extent in both absorbed and unabsorbed sera.

That portion of the Group II (F) bleeding kept, *as serum*, in the cold shows little depreciation in its agglutinating capacity.

It can be stated, then, that both antigen and agglutinin continue to be maintained for months in diagnosable form in dried blood specimens, and that probably in those blood-stains which have suffered most from adverse conditions, the absorption of a Group IV serum with the insoluble residue obtained when the dried blood is reconstructed will be the more successful diagnostic method.

With regard to the keeping properties of hæmagglutinating serum for diagnostic purposes, it may be mentioned here that the addition of 0·5 per cent. phenol, as is customary in the case of bacterial agglutinating sera, is not to be recommended. There is evidence that under the influence of the preservative the titre tends to sink.

Kolmer (6), working with serum, *not whole blood*, has found that samples dried on filter-paper deteriorate after 3 days in the cold room—how markedly one cannot tell, as the results given are qualitative only and not quantitative.

SUMMARY.

The possibility of grouping dried human blood specimens by reconstructing the serum for agglutination and using the undissolved residue for absorption has been demonstrated. Forensically the test would be of most importance when proving dissimilarity between two specimens alleged to be derived from the same source. To prove their similarity would probably only be to furnish circumstantial evidence of more or less value according to the group in question, and the frequency of the occurrence of that group in the population concerned.(7)

The Landsteiner theory that two substances, "A" and "B," with their corresponding agglutinins, "a" and "b," are concerned in the isoagglutination of human bloods has been confirmed by absorption tests.

REFERENCES.

1. Moss, W. L.—"Studies on Isoagglutinins and Isohemolysins," *Johns Hopkins Hosp. Bull.*, 1910, **21**, 63.
2. Landsteiner, K.—"Ueber Agglutinations-erscheinungen normalen menschlichen Blutes," *Wien. klin. Wochenschr.*, 1901, **14**, 1132.
3. Jansky, J.—"Hæmatologische Studien bei Psychotikern," reviewed in *Jahresb. f. Neurol. u. Psychiat.*, 1907, 1092.
4. Von Dungern, E., and Hirschfeld, L.—"Ueber gruppenspezifische Strukturen des Blutes," *Zeitschr. f. Immunitätsforsch.*, Orig., 1911, **8**, 526.
5. Happ, W. M.—"Appearance of Isoagglutinins in Infants and Children," *J. Exper. Med.*, 1920, **21**, 313.
6. Kolmer, J. A.—"The Influence of Desiccation upon Natural Hemolysins and Hemagglutinins in Human Sera," *J. Immunol.*, 1919, **4**, 393.
7. Hirschfeld, L. and H.—"Serological Differences between the Blood of Different Races," *Lancet*, 1919, **2**, 675.

THE PRESENCE OF HÆMOLYTIC SUBSTANCES IN HUMAN URINE.

ERIC PONDER, M.B.,

Assistant. Physiology Department. University of Edinburgh.

Received for publication November 6th, 1920.

In a paper published in 1915, C. S. McKee gave the results of his observations of the occurrence of hæmolytic substances in human urine. For the details of his technique, and for his results, readers are referred to his paper (¹).

The writer was so placed as to have at his disposal a large number of cases of various forms of insanity, from which it was possible to select a considerable number who were in good health, and none of whom suffered from any of the diseases in which the presence of hæmolytic substances in the urine has been noted.

OBSERVATIONS ON THE FREQUENCY OF THE OCCURRENCE OF HÆMOLYTIC SUBSTANCES IN THE URINE.

(A) The hæmolytic titre of the urine was estimated in 30 cases; of these, 20 suffered from epilepsy and 10 from senile dementia. All were in good health. The technique of McKee was closely followed, except that, for his method of measuring quantities by drops, measurements in fractions of c.c. were substituted, on the basis that one drop from a capillary such as he described equals about ·03 c.c. The titre of a urine was taken as the minimum quantity which would completely hæmolyse 1 c.c. of the standard blood suspension, used by McKee, when the mixture was subjected to a temperature of 37° C. for 2½ hours. In each case the urine was examined in this way daily, the titrations being done at the same time each day (8 a.m).

The results may be expressed as follows:

In each of the 30 cases, at one time or another, hæmolytic activity was observed in the urine, no case giving persistently non-hæmolytic urine throughout the period during which it was under observation.

The daily variation in the hæmolytic titre was as a rule very marked; often for days the urine would fail to hæmolyse, and then, without an apparent cause, highly hæmolytic urine would be excreted.

The frequency with which the various titres of urine were met with is shown by the following table:

Cases.	Observations.	Percentage of total observations.					
		Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
8	336	54	15	13	10	2	6
12	336	75	13	10	1	·5	·5
10	210	71	11	4	4	8	2
Total 30	882	66·6	13	9	5	3·5	2·9

Each of the first group was examined on 42 consecutive days, each of the group of 12 cases on 28 consecutive days, and each of the group of 10 cases on 21 consecutive days.

Thus, out of 882 observations, hæmolytic substances were observed to be present on 33·3 per cent. of the occasions on which estimations were made, while no hæmolytic substance was detected on 66·6 per cent. of these occasions.

(B) Before making more extensive observations, it was considered advisable to investigate the technique used, to see if it gave the maximum number of positive results. Four points were specially investigated:

(1) *The time factor.*—The effect of the length of time in the incubator on the number of positive results obtained was investigated thus: 1000 urines, all known to be hæmolytic, were titrated by adding to various quantities of each urine 1 c.c. of the blood suspension, this being of a strength similar to that used above, but made with thrice washed cells. After one hour in the incubator at 37° C. the tubes were shaken; they were then returned to the incubator. After two hours the process was repeated, and the number of tubes showing hæmolysis noted. In a similar way the number of tubes showing hæmolysis at the end of 2½, 3, 4, 5 and 6 hours was ascertained, these numbers being expressed as a percentage of the total number of positives—1000.

Percentage positive after—					
2 hours.	2½ hours.	3 hours.	4 hours.	5 hours.	6 hours.
2·1	32·3	60·6	97	99·3	100

(No. of positives = 1000.)

It is, then, obvious that to state the number of urines which show hæmolytic activity at the end of 2½ hours is not to state the number which contains hæmolytic substances, but only about one-third of that number. This is one more instance of the neglect of the all-important time factor, and of the fallacy of assuming an end-point which does not exist experimentally.

(2) *Washing of cells.*—From the formulæ for the washing of precipitates (²) the quantity of serum left in McKee's suspension may be calculated; it will be found to be small. Since, as the result of research not included in this article, it has been found that this amount of serum may influence quantitative estimations considerably, the blood suspension used in the series of titrations below was made with thrice-washed cells, the resulting suspension being of greater sensitiveness than the suspension of once-washed cells.

(3) *Loss of hæmolytic power.*—The results of an investigation into the loss of hæmolytic power on standing may be summarised thus: There is a similarity in the way in which hæmolytic urines behave on standing, the difference being one more of degree than of kind. As a rule, a hæmolytic urine shows no loss of power in the first 12 hours after being passed. After this period loss of power is liable to occur; it commences in some urines sooner than in others, but proceeds with great rapidity once it has begun. It is occasionally, but not frequently, observed that a urine increases in hæmolytic power during the first few hours after being passed; this fact is recorded by McKee. It is therefore desirable to estimate the titre of a urine about 3 to 6 hours after it is passed.

(4) *Effect of temperature.*—There is no reason why this reaction should take place at body temperature only. Like most reactions, it is accelerated by high temperatures and retarded by low ones. The reaction occurs at 45° C., even more rapidly than at 37° C.; it also occurs at 30° C., the time taken being much longer. It is not proposed to fully discuss this question in this paper; it will be sufficient to note that subjection to incubator temperatures is not essential, but merely convenient.

As a result of these observations on technique, it was found necessary to devise a new method, of greater sensitiveness, for deciding the presence or absence of hæmolytic substances. The following was adopted:

Blood suspension.—To a tube containing 15 c.c. of citrated saline, add ·45 c.c. of blood drawn freely from the finger. Wash the cells thrice, using each time 15 c.c. of fluid, but using normal saline after the first washing. The washed cells are finally added to 50 c.c. of saline. The suspension is to be used soon after preparation.

Urines.—These are tested within a few hours of being passed. For each urine to be tested put up a series of 6 tubes containing 0·45 c.c., 0·36 c.c., 0·30 c.c., 0·21 c.c., 0·12 c.c., 0·06 c.c. of urine. To each tube add 1 c.c. of the blood suspension. Incubate for 4 hours at 37° C., shaking thrice in this time. Complete or marked hæmolysis to be considered positive. A few controls should always be put up.

(C) With this technique, a number of specimens of urine were examined, with a view to ascertaining the frequency of the appearance of the hæmolytic quality. These observations were made in three groups:

300 cases, examined on each of 14 consecutive days.—All were cases of various forms of insanity; all were in good health, cases with "organic" lesions being avoided. The urines were examined at the same time each day, some at 8 a.m. and some at 6 p.m. The results may be thus summarised:

No case gave negative results on more than 5 occasions out of the 14. Many cases gave no negative reading at all during the 14 days. There was a considerable diurnal variation in titre. Expressed in a tabular form, similar to that used in (A), we have the results:

		Percentage of total observations.					
Cases.	Observations.	Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
200	2800	6·5	3·5	8	21	34	27

Thus out of 2800 titrations, hæmolytic activity was observed in 93·5 per cent., while only 6·5 per cent. were negative.

300 cases, observed on one occasion only.—These cases were all those of insane persons, all in good health. This series was examined from two points of view: (*a*) The numbers which showed various degrees of hæmolytic activity at the end of 4 hours; and (*b*) the numbers which showed hæmolytic activity at the end of 2½ hours.

(*a*) The results may be tabulated:

		Percentage of total observations.					
Cases.	Observations.	Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
300	300	3·6	1·6	1·3	10·3	23	60

Or, out of 300 cases, 96·4 per cent. showed the presence of hæmolytic substances; in 3·6 per cent. these substances were absent. The 11 negatives were examined on subsequent days; none remained negative, but all showed hæmolytic activity.

(*b*) At the end of 2½ hours, of the 300 cases 47 per cent. showed hæmolytic activity, while 53 per cent. showed no activity. Combining the results of the 200 cases and of the 300 cases, we may express the result:

		Percentage of total observations.					
Cases.	Observations.	Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
500	3100	5·1	2·6	4·7	15·5	28·5	43·5

Out of the 3100 titrations, 94·9 per cent. were positive, while 5·1 per cent. were negative.

The urines of 25 normal persons, titrated daily for 28 days.—The results may be summarised thus: No case showed a persistent negative reaction, each case showing, at one time or another, the presence of hæmolytic action. In tabular form:

		Percentage of total observations.					
Cases.	Observations.	Neg.	·45 c.c.	·36 c.c.	·30 c.c.	·21 c.c.	·12 c.c. or less.
25	700	5	3	13	9	25	45

Out of 700 titrations, 5 per cent. were negative and 95 per cent. positive.

From the observations on the urines the following conclusions may be drawn: Marked hæmolytic activity being observed at one time or another in 100 per cent. of the urines of insane persons examined and in 100 per cent. of the urines of normal persons examined, it is probable that the presence of hæmolytic action in the urine is a normal occurrence. In all probability it is due to a normal known urinary constituent.

THE PHYSICAL CONDITION OF HÆMOLYTIC URINE.

Two points suggest themselves for consideration:

(1) *The reaction of the urine.*—In all cases the reaction of the urine was tested qualitatively, while in a series of 50 cases the acidity was estimated by titration. The results may be summarised by saying that a hæmolytic action

may be shown by either an alkaline or an acid urine, although the majority of the hæmolytic urines examined were acid in reaction. The degree of acidity did not appear to exercise any effect. Certain of the persistently negative urines were alkaline; it was observed that an ammoniacal urine rarely produced hæmolysis. It has been pointed out (³) that solutions acid to P_H 5·1 produce hæmolysis; it is obvious that this has no bearing on the hæmolysis of urine (P_H 6 approximately) when such a urine is diluted 1 in 10.

(2) *The total solids.*—These are of importance, as they determine the tonicity of the urine. In a series of 50 specimens of urine, of various hæmolytic titre, the total solids were estimated; the osmotic pressure of the urine could approximately be calculated from this. It was found, in general, that urines with little hæmolytic power contained little solid, while highly hæmolytic urines tended to be more concentrated; the relation, however, did not seem to be a regular one. Many non-hæmolytic urines were found to be diluted specimens.

That the hæmolytic phenomenon observed in urine is not a result of a variation in tonicity is obvious from the following considerations: (1) That diluted specimens of urine which, when added to the saline suspension of blood, would tend to make it hypotonic, are, as a rule, non-hæmolytic, whereas urines which show hæmolytic power are, as a rule, those containing much solid, and which would tend to make the blood suspension to which they were added hypertonic; (2) that the addition of 0·1 c.c. of distilled water to 1 c.c. of the blood suspension will not cause hæmolysis, whereas the addition of 0·1 c.c. of a hæmolytic urine of a tonicity more equal to that of the blood will cause a rapid hæmolysis; (3) that the hæmolysis caused by urine is entirely inhibited by even a trace (0·005 c.c.) of blood-serum, or by the addition of lipoids, etc.

It is therefore necessary to consider that the hæmolysis is caused by some substance or substances, and not by a change in tonicity.

THE IDENTIFICATION OF THE HÆMOLYTIC SUBSTANCE.

Certain observations, which give an indication of the nature of the substance, may first be noted.

Raising a hæmolytic urine to a temperature of 100° seems to have no effect on its hæmolytic power; this temperature may be sustained for as long as an hour and a half without greatly reducing the activity of the urine. Further, the urine may be evaporated over a water-bath to dryness, and the residue treated with alcohol; the hæmolytic substance passes into the alcohol, which may be evaporated and the residue taken up with saline, giving a hæmolytic fluid.

In a similar way it may be shown that the substance is not soluble in ether, benzene or chloroform. It is obvious that it is fairly stable.

With a view to isolating the substance, two lines of research were followed: (*a*) a quantitative analysis of the constituents of hæmolytic and non-hæmolytic urines, and (*b*) an investigation into the hæmolytic activity of the various urinary constituents.

(*a*) Thirty urines were analysed, and at the same time their titres were estimated. Chlorides, phosphates, oxalates, sulphur (organic, inorganic and neutral), urea, creatin, creatinin, uric acid, purin bases, hippuric acid, ammonia and total nitrogen were estimated quantitatively. Phenol, cresol, indol, indoxyl, skatol, aromatic oxyacids, leucin, cystin, tyrosin and nucleo-albumens were estimated by less exact methods.

This investigation threw no light on the question, the excretion of none of these substances being parallel to the hæmolytic titre.

Bile-acids and urobilin were also estimated; regarding these a detailed note is required.

Bile-acids.—The test used was that of Oliver. The urines were diluted 1 in 3 before testing. As soon as the peptone solution had been added the tubes were examined for a cloud; they were then set aside for 15 minutes, and again examined. The intensity of the cloud, and the time which it took to appear, were thus under observation. The tubes were examined both by transmitted light and against a dead black ground. In all there were tested 100 urines of known hæmolytic titre. It was found, without exception, that the urines which exhibited most hæmolytic activity gave the densest and most immediate clouds, while weakly hæmolytic urines, and urines which were non-hæmolytic, gave either scarcely a trace of cloud in the acid peptone or no cloud at all. So close was the parallelism between the hæmolytic activity and the cloud production in peptone, that it was found quite easy to foretell with accuracy the results which would be given by the titration of the hæmolytic power by examining the power of the urine to precipitate peptone.

Urobilin.—This was estimated by the spectroscope. The quantity of urobilin and the hæmolytic power ran roughly, but not absolutely, parallel.

(*b*) An investigation into the hæmolytic powers of the various urinary constituents did not reveal any substance of sufficient hæmolytic power to be considered as the cause of this occurrence, with the exception of the bile-acids and substances closely related to them. The bile-acids and their salts are very highly hæmolytic, a 1 in 10,000 solution of sodium taurocholate in saline, for instance, producing complete hæmolysis of a quantity of blood suspension considerably stronger than that used in the experiments above at 37° C., and in 4 hours. When this intensely hæmolytic power is considered, even a trace of such a substance in urine would be sufficient to confer on the urine a hæmolytic power.

That traces of bile-acids and their salts may occur in normal urine has been noticed by various observers [4]. The results of the test applied for bile-acids, which are recorded above, point to this fact, no other substance which gives the peptone precipitation being known to occur in urine. It will also be observed that the results of attempts at extraction of the hæmolytic substance by various solvents show the substance to have a solubility similar to that of the bile-acids and their salts. It is further the case that these substances hydrolyse after a period of time, and on prolonged heating; thus, if the bile-acids were the hæmolytic agents, the loss of hæmolytic power on standing and the loss of power after prolonged heating would be explained.

It is therefore suggested that the hæmolytic activity of urine is due to traces of bile-acids or of their salts; that this occurrence is physiological, and not necessarily connected with any abnormal process.

A note may be added on the inhibition of the hæmolytic action of urines which is caused by certain substances:

(*a*) *Sodium bicarbonate*, or a dilute alkali, will inhibit the hæmolytic action of urine; this fact was recorded by McKee.

(*b*) Minute quantities of serum have the same effect, but in a much greater degree, *e.g.* 0·005 c.c. of a certain normal serum inhibited the action of a urine whose titre was 0·27 c.c. Sera differ much in their inhibitory power; the question will not be dealt with fully in this paper.

(*c*) *Cerebro-spinal*, *ascitic* and other body-fluids have the same effect, but in a less degree.

(*d*) *Lipoids*, such as cholesterol and lecithin, and also certain fats, exert a powerfully inhibitory effect.

It is noteworthy that these same substances act as inhibitory agents to the hæmolytic action of the bile-acids and their salts, and that their inhibitory action on hæmolysis caused by urine is explicable on the ground that this hæmolysis is produced by the bile-acids and their salts.

This investigation is being continued in the Chemical Physiological Laboratory of Edinburgh University.

The writer desires to express his indebtedness to Prof. Sir Edward Sharpey Schafer for the interest which he has taken in this investigation, and for the encouragement and the opportunities which he has given. Dr. N. R. Phillips, Superintendent of the Monmouthshire County Asylum, Abergavenny, kindly gave every facility for the carrying out of the initial part of this investigation. To him especial thanks are due, and also to Dr. W. W. Taylor for the valuable advice and direction which he has given.

SUMMARY.

Hæmolytic activity was observed as a regular occurrence in the urines of insane persons, when tested for by a sensitive method. Such activity was also found to occur in the urines of healthy persons. It was found that in previous work on this subject certain important factors were overlooked. The hæmolytic substance appeared to be a stable chemical compound, capable of being extracted from the urine with various solvents. Its activity was inhibited by various substances, notably blood serum. There seems to be sufficient evidence to justify the suggestion that the hæmolytic power of urine depends on its containing minute traces of bile acids or their salts, and that the occurrence of hæmolytic activity in urine is physiological.

REFERENCES.

1. McKee, C. S.—"On the Hæmolytic Action of the Urine in Certain Conditions," *Brit. Med. J.*, 1915, **2**, 596.
2. Ostwald, W.—'Foundations of Analytical Chemistry,' 1908, p. 17.
3. Cole, S. W.—'Practical Physiological Chemistry,' 1915, p. 239.
4. Dragendorff.—*Zeitschr. f. anal. Chem.*, 1872, **11**, 467.

THE SACHS-GEORGI SYPHILIS REACTION AND ITS RELATION TO THE WASSERMANN REACTION.

TENJI TANIGUCHI.

From the Pathological Department of the University and Western Infirmary, Glasgow.

Received for publication January 6th, 1921.

Porges and Meier (1) and others have found that syphilitic sera caused precipitation of lecithin from a watery emulsion, as well as of sodium glycocholate. The phenomenon of precipitation has been held to be an essential antecedent to the absorption of complement in the Wassermann reaction: thus a number of precipitation reactions have been advanced as substitutes for the Wassermann reaction, the precipitable substances being lipoids or other hydrophilic colloids, or the attempt has been made to cause precipitation of the globulin of the serum, since it is agreed by numerous workers that the reacting power of syphilitic serum resides in the globulin fraction. But none of these reactions has come into practical use, as the general results did not run parallel either with the Wassermann reaction or with clinical diagnosis. Lately Sachs and Georgi (2, 3) have devised an improved method for detecting syphilitic infection by the formation of precipitates when the sera are mixed with cholesterolised alcoholic tissue extracts. The results show close parallelism to those of the Wassermann test and also correspond well with the clinical diagnosis. As this test is easier to carry out, it has been recommended by a number of workers as a substitute for the Wassermann reaction. It has been shown that physical conditions which are known to affect the Wassermann reaction exert a similar influence on Sachs and Georgi's reaction, *e.g.* the degree of dilution of the organ extract, the addition of cholesterol to the extract and the turbidity of the emulsion (Sachs and Georgi); also the reaction of the medium and the temperature, (Georgi) (4, 5). Accordingly, Sachs and his associates believe that the two reactions depend on the same principle. It has been found by a number of workers, however, that the two reactions are not strictly parallel either quantitatively or qualitatively; thus certain sera react positively to the Wassermann test but show a negative result in the Sachs-Georgi reaction, and *vice versâ*. As was pointed out by Neukirch (6) and Stilling (8), there often occurs non-specific precipitation when the mixtures of lipoid emulsion and human serum are allowed to stand at lower temperatures; accordingly Sachs and Georgi (1920) have modified their original procedure, and now keep the mixture for 18 to 20 hours at 37°C., making the reading immediately at the end of this period ("incubator method"). In view of the importance of this reaction an investigation has been made as to the relationship between the Sachs-Georgi and the Wassermann reactions.

PARALLELISM BETWEEN THE WASSERMANN AND SACHS-GEORGI REACTIONS.

The Wassermann reactions have been carried out according to the following method employed in this laboratory (9):

Antigen.—1 : 30 dilution of cholesterolised human heart alcoholic extract. *Hæmolytic system.*—3 per cent. suspension of ox corpuscles sensitised with excess of corresponding immune body (5 M. H. D.).

Procedure.—To 0·5 c.c. of antigen, 0·05 c.c. of heated serum (30 minutes at 56°C.) and then 2, 4, 6 M. H. D. of complement were added respectively for the first, second and third tubes. After the tubes had been incubated at 37°C. for 1·5 hours, 0·5 c.c. of sensitised ox corpuscles was added to each. Results were recorded after further incubation at 37°C. for one and a quarter hours and standing overnight at room temperature. *Controls.*—Serum alone, emulsion alone, also strong positive, weak positive and known negative sera. The results were recorded according to the method of Browning and Kennaway (10).

For carrying out the Sachs-Georgi reaction, the incubator method of Sachs and Georgi has been followed (3) except as regards the amount of serum used. According to Sachs and Georgi, one tube containing 0·1 c.c. of serum was used for the main test, but with this amount of serum a zone phenomenon occurs sometimes with extremely strong positive sera, as illustrated in Table I. It was also observed that a suspicious cloudiness may develop with certain negative sera in this amount; thus the one tube method with 0·1 c.c. of serum is not advisable in carrying out the reaction. Accordingly, for the main test at least two tubes containing 0·1 and 0·05 c.c. of serum were employed.

Antigen.—1·0 c.c. of 10 per cent. alcoholic human heart extract, as used in the Wassermann reaction, was diluted with 0·5 c.c. of absolute alcohol (practically equivalent to Sachs and Georgi's final dilution of extract); to one part of this diluted extract 0·05 c.c. of 1 per cent. alcoholic solution of cholesterol was added. Then 1 part of the cholesterolised extract was mixed with 5 parts of normal saline by the "double" method (viz. 1 c.c. each of extract and saline were mixed rapidly; after the mixture had stood for at least 15 minutes at room temperature, a further amount of 4 c.c. physiological saline was added rapidly). *Sera* (within 7 days after withdrawal of the blood) were tested after standing at least 3 hours at room temperature subsequent to heating 30 minutes at 56° C. (as recommended by Münster).

Procedure.—To each of two tubes containing 0·5 c.c. of emulsion, a volume of 1·0 c.c., consisting respectively of 0·1 and 0·05 c.c. serum diluted with saline, was added and mixed by slight shaking; the tubes were then incubated overnight (18 to 20 hours) at 37° C. *Controls* were similar to those used in the Wassermann reaction, for the serum control 0·1 c.c. of serum was used with 1·5 c.c. of saline containing the same amount of alcohol as in the test series (in the absence of alcohol a growth of bacteria often may be mistaken for precipitate). Results were designated −, ±, +, ++, +++, ++++, respectively, indicating negative to strong positive precipitation.

In determining the result of the reaction, the presence or absence of precipitate in the second tube (containing 0·05 c.c. of serum) was principally taken into account (see above). But when this tube showed merely a suspicious reaction, the result in the tube containing 0·1 c.c. serum was compared with the controls; if this tube showed precipitation while the same tube in the negative control showed none, a positive result was recorded.

TABLE I.—*Illustrative Series showing Parallelism between Wassermann and Sachs-Georgi Reactions.* (The 7 sera detailed here were included in a series of 35, tested all on the same day.)

No. of serum.	Wassermann reaction.					Sachs-Georgi reaction.				
	Doses of complement.				Result.	Amounts of serum in c.c.				Result.
	2	4	6	serum control 2		0·1	0·05	0·03	serum control 0·1	
1	c	c	c	c	−	−	−	−	−	−
2	o	o	o	c	+	++++	+++	++	−	+
3	o	o	o	c	+	++	+	+	−	+
4	tr	m	jc	c	weak +	+	±	−	−	weak +
5	ac	c	c	c	−	±	−	−	−	−
6	ftr	tr	jc	c	weak +	*±	±	−	−	suspic.
7	o	o	o	c	+	†+	+++	++++	−	+
A Controls	d	vm	jc	c	weak +	+	±	−	−	weak +
B	o	o	o	c	+	++++	++++	++	−	+
C	c	c	c	c	−	−	−	−	−	−

Controls: For Wassermann reaction, M.H.D. of complement with 0·5 c.c. 3 per cent. ox corpuscles sensitised with 5 M.H.D. of immune body = 0·0075 c.c.; emulsion alone with two doses of complement = c; emulsion control for Sachs-Georgi reaction = no precipitation.

In the tables † indicates zone phenomenon; * indicates discrepancy. The degrees of hæmolysis in the Wassermann reaction are designated as follows: c = complete, jc = just complete, m = marked, d = distinct, tr = trace, ftr = faint trace, o = none. In the Sachs-Georgi reaction, the *plus* signs are in proportion to the degree of precipitation; − = no precipitation.

It is known that alcoholic extract of guinea-pig heart sometimes causes a false positive Wassermann reaction when used as antigen. The author (11) has demonstrated that this is due to the interaction between heterophile antibody contained in the particular normal human sera (normal sheep hæmolysin) and heterophile antigen contained in the guinea-pig tissue extract. An analogous fallacy in the Sachs-Georgi reaction has also been observed.

According to Sachs and Georgi, the precipitate obtained by the incubator method is less marked than that by the older method; but the author has observed that the results in the former case can be intensified by allowing the tubes to stand for 15 to 30 minutes at room temperature after removal from the incubator; thus it is advisable in suspicious cases to repeat the reading after 30 minutes, in addition to the immediate reading after incubation; but, of course, the negative control must be observed likewise.

The results of the two reactions show very close parallelism; thus out of 296 cases examined on thirteen different days, 270 (91·2 per cent.) gave the same result, comprising 104 positives, 164 negatives and 2 suspicious; 26 cases (8·8 per cent.) showed discrepancies as detailed in Table II. None of the discrepancies can be regarded as serious, but it is to be noted that the largest proportion occurs in cases which are suspicious to the Wassermann test but negative to the Sachs-Georgi reaction.

TABLE II.—*Discrepanies between Sachs-Georgi and Wassermann Reactions in a total of* 296 *cases.*

	Results.					
Wassermann reaction	Weak positive		Suspicious		Negative	
Sachs-Georgi reaction	—	?	—	weak +	weak +	?
Total 26	0	4	12	4	0	6

These results accord well with those of other investigators; thus the reactions were parallel in 94·94 per cent. out of 2770 cases (Sachs and Georgi); in 95·2 per cent. out of 165 (Plaut), in 85 per cent. out of 549 (Schröder). It seems unlikely that the discrepancies are due to any essential difference in the nature of the two reactions. In general, differences have been observed only in the case of weak or doubtful reactions. It is clear that the Sachs-Georgi reaction exhibits with certain sera a result intermediate between positive and negative, just as occurs with the Wassermann reaction (see Browning and Kennaway). Accordingly, here also the criterion of positive and negative stands on an empirical basis. As regards the relative advantages and disadvantages of the two tests from the practical standpoint, the Sachs-Georgi reaction suffers from the greater difficulty of distinguishing between negative and suspicious or weakly positive reactions, since slight differences in the state of aggregation of the suspension cannot be sharply distinguished; on the other hand, there is the advantage that the variable factor of the complement-containing serum is eliminated.

When the amount of complement fixed by the standard amount of serum in the Wassermann reaction is compared with the concentration of serum required to cause precipitation in the Sachs-Georgi test, it seems that a very close parallelism exists (Table III).

TABLE III.—*Parallelism between the Degree of Complement-fixation in the Wassermann Reaction and of Precipitation in the Sachs-Georgi Test.*

Series A.

For the Sachs-Georgi reaction in series A, the mixture of alcoholic lipoid and saline solutions was effected rapidly and all at one time in order to produce less turbid emulsions and so to facilitate accurate reading (the actual amount of precipitate being somewhat diminished thereby); in series B the antigen was diluted as described in the text. For the Wassermann reaction in both series cholesterolised extract was diluted in the proportion of 1 : 15 with saline. Excepting these modifications, the procedures for both reactions are the same as already described.

No. of serum.	Sachs-Georgi reaction: precipitate obtained with the following amounts of serum in c.c.—				Wassermann reaction: doses of complement fixed.
	0·1	0·05	0·03	0·01	
1	+ + + +	+ + + +	+ + + +	+ + +	30 +
2	+ +	+	±	—	20 to 25
3	+	+	±	—	15 „ 20
4	±	—	—	—	8 „ 10
5	±	—	—	—	6 „ 8
6	±	—	—	—	4 „ 6

In this series two other sera gave the same results as No. 3, and one other the same as No. 4.

Series B.

No. of serum.	Sachs-Georgi reaction: precipitate obtained with the following amounts of serum in c.c.—				Wassermann reaction: doses of complement fixed.
	0·1	0·05	0·03	0·01	
1	+ +	+ + + +	+ + + +	+ + + +	40 +
2	+ + +	+ + + +	+ + + +	+ +	35 to 40
3	+ + + +	+ + +	+ + +	+	25 „ 30
4	+ + +	+ + +	+	±	15 „ 20
5	+ + +	+ +	+	—	12 „ 15
6	+	±	—	—	4 „ 6

In this series one other serum gave the same result as No. 4, and one the same as No. 6.

THE COMPARATIVE INTENSITY OF THE SACHS-GEORGI AND THE WASSERMANN REACTIONS WITH HEATED AND UNHEATED SERA; THE INFLUENCE OF COMPLEMENT ON THE PRECIPITATION REACTION.

It has been pointed out by Georgi (5) and by Münster (14) that, in contrast to the Wassermann reaction, unheated sera give rise to less intense precipitation in the Sachs-Georgi reaction than occurs after inactivation at 56° C. This has been corroborated by Neukirch (7), who found also that fresh guinea-pig's serum possesses the property of diminishing precipitation when added to the mixture of lipoid emulsion and heated serum. Neukirch observed further that it causes solution of the precipitate, after it has been formed by the specific combination; the author has also found independently that fresh guinea-pig's serum lyses the precipitate obtained by adding the lipoids of heterophile antigen to the corresponding antibody. Again, these properties of guinea-pig's serum should be attributed to the action of complement; thus heated guinea-pig's serum is inactive in this respect, and lysis of the precipitate never takes place with fresh serum at 0° C. These differences in the behaviour of heated and unheated sera in the two reactions do not necessarily exclude the possibility of their being due to the same constituent. (See Table IV.)

TABLE IV.—*Lytic Effect of Fresh Guinea-pig's Serum on the Precipitate Formed in the Sachs-Georgi Reaction.*

A series of tubes containing lipoid emulsion and varying amounts of heated syphilitic sera were incubated at 37° C. overnight (Sachs-Georgi reaction): then 0·2 c.c. of guinea-pig's serum was added to each tube; in series (*a*) fresh (M.H.D. for 0·5 c.c. of 3 per cent. ox corpuscles sensitised with 5 M.H.D. of immune body =0·0075 c.c.), (*b*) heated for 30 minutes at 56° C. (0·2 c.c. caused no lysis of the test corpuscles), and the mixtures were kept at 37° C. for three hours. The results were as follows:

	Precipitate obtained with the following amounts of serum in c.c.		
	0·02	0·01	0·005
Before addition of guinea-pig serum	+ + + +	+ + +	+
After addition of fresh guinea-pig serum	+ + +	+	—
After addition of heated guinea-pig serum	+ + + +	+ + +	+

THE NATURE OF THE PRECIPITABLE SUBSTANCE IN THE SACHS-GEORGI REACTION.

Highly divergent views are held regarding the part played by the lipoid emulsion in the formation of precipitates with syphilitic serum. Georgi (5) believes that the precipitate consists mainly of globulin derived from the syphilitic serum owing to the fact that it never redissolves completely in ether or alcohol. As is shown in the following example, however, the precipitate contains practically the total amount of lipoids of the emulsion. Thus when a lipoid emulsion is precipitated as completely as possible by adding a suitable amount of syphilitic serum until the supernatant fluid appears quite clear, it is found that an alcoholic extract obtained by treating the precipitate with an amount of alcohol equivalent to that originally present is practically as efficacious as the original extract for producing hæmolysis with cobra-venom; whereas the clear fluid, obtained after centrifuging off the precipitate, does not exhibit this property, except to a minimal degree.

Example.—To 3 c.c. of the same extract as was used in the Sachs-Georgi reaction, but without cholesterol, 15 c.c. of normal saline were added slowly to produce an emulsion of maximum turbidity, and then 2 c.c. of syphilitic serum (amount of complement fixed by 0·05 c.c. of this serum with 0·5 c.c. of the lipoid emulsion diluted 1 : 15 = 40 + M. H. D. ; maximum precipitating dose for 0·5 c.c. of test lipoid emulsion=0·05 c.c.). The volume was then made up to 50 c.c., and 10 c.c. of the mixture were placed in a series of tubes, which were allowed to stand in the incubator at 37°C. for 24 hours, being shaken thoroughly at intervals. Thus a bulky precipitate formed and the supernatant fluid became clear. The precipitate was centrifuged at high speed and washed twice with salt solution (but a certain amount of the precipitate was lost owing to the difficulty in obtaining complete sedimentation). Finally the precipitate was extracted with 3 c.c. (original volume) of alcohol for 48 to 72 hours at room temperature, being shaken several times. The filtered alcoholic extract was tested for lytic power with cobra-venom with the following result :

Lytic dose for 1·0 c.c. of 5 per cent. ox corpuscles + cobra-venom 1 : 1000 in the different series :

(*a*) With emulsion of extract from precipitate (=1 : 8 dilution of original alcoholic solution, slowly mixed) = between 0·1 and 0·15 c.c. ; [(*b*) with supernatant fluid (= 1 : 17 dilution of original alcoholic solution) 0·6 c.c.=trace of lysis] ; (*c*) with emulsion of original extract (1 : 8 dilution, slowly mixed) = between 0·08 and 0·1 c.c.

CONCLUSIONS.

296 human sera were examined by the " incubator method " for Sachs and Georgi's reaction and simultaneously for the Wassermann reaction. 270 cases (91·2 per cent.) gave the same result with the two reactions, comprising 104 positives, 164 negatives and 2 suspicious ; 26 cases (8·8 per cent. showed discrepancies. But none of the discrepancies can be regarded as serious, because the largest proportion (12 cases out of 26) occurs in the cases which are suspicious to the Wassermann test but negative to the Sachs-Georgi reaction, due apparently to the lesser delicacy of the latter reaction, owing to the difficulty of detecting slight precipitation. In general, differences have been observed only in the case of weak or doubtful reactions. Accordingly, it seems unlikely that the discrepancies are due to any essential difference in the nature of the two reactions.

When the amount of complement fixed by the standard amount of serum in the Wassermann reaction is compared with the concentration of serum required to cause precipitation there is also seen to be a close parallelism.

The difference in the behaviour of unheated sera in the two reactions does not necessarily exclude the possibility of their being due to the same constituent, as there is evidence that the thermolabile constituent of the serum, which is not a specific constituent in the syphilis reaction, affects differently the result in the two reactions.

The precipitable substance in the Sachs-Georgi reaction consists mainly of the lipoids contained in the emulsion.

REFERENCES.

1. **Porges, O., and Meier, G.**—"Ueber die Rolle der Lipoide bei der Wassermann'schen Syphilis-Reaktion," *Berlin. klin. Wochenschr.*, 1908, **45**, 731.

2. Sachs, H., and Georgi, W.—"Zur Serodiagnostik der Syphilis mittels Ausflockung durch cholesterinierte Extrakte," *Med. Klin.*, 1918, **14**, 805.
3. *Idem.*—"Beiträge zur Serodiagnostik der Syphilis mittels Ausflockung durch cholesterinierte Extrakte," *Arb. a. d. Inst. f. exper. Therap. . . . Frankfurt a/M.*, 1920, **10**, 5.
4. Georgi, W.—"Die Bedeutung der Extraktbeschaffenheit für die Ausflockung des syphilitischen Blutserums," *Zeitschr. f. Immunitätsforsch.*, Orig., 1918, **27**, 518.
5. *Idem.*—"Studien über Serum-Ausflockung bei Syphilis," *Biochem. Zeitschr.*, 1919, **93**, 16.
6. Neukirch, P.—"Ueber den Einfluss der Temperature und anderer Faktoren auf die Serumausflockung bei Syphilis," *Arb. a. d. Inst. f. exper. Therap. . . . Frankfurt a/M.*, 1920, **10**, 47.
7. *Idem.*—"Studien über die Sachs-Georgische Ausflockungsreaktion," *Zeitschr. f. Immunitätsforsch.*, Orig., 1920, **29**, 498.
8. Stilling, E.—"Zur Frage der Specificität beim serologischen Luesnachweis mittels Ausflockung nach Sachs und Georgi," *Med. Klin.*, 1920, **16**, 41.
9. Browning, C. H., and Watson, D.—'Venereal Diseases,' London, 1919, p. 302 (Oxf. Med. Public.).
10. Browning, C. H., and Kennaway, E. L.—"Suggestions for a New Criterion of a Positive Wassermann Reaction based on an Analysis of 2334 Quantitative Tests," *J. Hyg.*, 1920, **19**, 87.
11. Taniguchi, T.—"A Source of Fallacy in the Wassermann Reaction depending on Heterogenetic Antibody in Human Serum," *J. Pathol. and Bacteriol.*, 1920, **23**, 368.
12. Schröder, H.—"Ueber Ergebnisse der Ausflockungsreaktion nach Meinicke und Sachs-Georgi für die serologische Luesdiagnostik," *Med. Klin.*, 1919, **15**, 515.
13. Plaut, F.—"Die Sachs-Georgische Ausflockungsreaktion für Syphilis," *Zeitschr. f. d. ges. Neurol. u. Psychiat.*, Ref., 1919, **17**, 383.
14. Münster, M.—"Untersuchungen und Erfahrungen mit der Sachs-Georgischen Reaktion zur Serodiagnostik der Syphilis," *München. med. Wochenschr.*, 1919, **66**, 505.

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

THE QUESTION OF SEROLOGICAL RACES OF *V. CHOLERÆ* AND THE RELATION OF SOME OTHER VIBRIOS TO THIS SPECIES.

S. R. DOUGLAS, I.M.S. (ret.), M.R.C.S.,

Acting Director, Bacteriological Department, National Institute for Medical Research, Hampstead, London.

Received for publication December 11th, 1920.

At one time it was almost universally believed that epidemic cholera was caused by a number of races of vibrios which could be separated from one another by small cultural and morphological differences; all, however, were included under the name of *V. choleræ.*

It was early recognised that certain serum tests—Pfeiffer's reaction, and agglutination tests—were the most certain methods of ascertaining whether or no the vibrio in question was capable of causing true epidemic cholera. However, before these tests were adjudged the necessary criterion of classing a vibrio as a true member of the species *V. choleræ*, a number of organisms had been isolated from various sources, such as well-water, *e. g. V. nasik*, or from the fæces of human beings not suffering from cholera—such as the various El Tor vibrios—which were culturally and morphologically very similar to the true *V. choleræ.*

These organisms were later investigated by serum tests, and somewhat confusing results were obtained by different workers.

More recently, from sporadic outbreaks of a cholera-like disease, usually recognisable from true epidemic cholera by the restricted area of the epidemic, milder symptoms and lower case mortality, vibrios have been isolated which in morphology, some cultural characters, and in their pathogenicity for the usual laboratory animals, resemble *V. metchnikovi* almost as closely as *V. choleræ.* Serologically, these organisms have been described as quite distinct from the true *V. choleræ* and have been separated into a number of serological races. This disease has been denominated paracholera and the organisms as *V. paracholeræ* A, B, etc., according to the serological grouping.

Under these circumstances, and in view of the work which has been carried out by Andrewes and Inman on the *B. dysenteriæ* (Flexner) group of organisms, it was decided to ascertain if a number of strains of *V. choleræ* showed any serological differences, and also to reinvestigate the serological characters of certain of the vibrios concerning which a variety of opinions have been expressed.

Nine strains of *V. choleræ* were obtained, eight having been collected at various times and from various sources by the Pathological Department of the Royal Army Medical College. Of these, one strain at least was obtained from Galicia by J. Freeman in 1915; three strains were originally obtained from the Pasteur Institute at Paris; the exact source of the remaining four was not known. The ninth strain was one which the Italian Government employed in the manufacture of the anti-cholera vaccine used by the Italian army during the late war.

From this last strain a vaccine was prepared and a series of doses of from 200 to 500 million vibrios was given intravenously to a rabbit. When preliminary tests showed that the serum had an agglutinating titre of about 800 to 1000, the animal was bled from the ear-vein, and the serum thus obtained stored in hermetically sealed small test-tubes, each containing about 3 c.c.

The first experiment was made to ascertain if, using this serum, emulsions from nine strains of *V. choleræ* were agglutinated to approximately the same titre. The emulsions were made by planting each strain into peptone water, incubating for from eighteen to twenty hours at 37° C. and then diluting the cultures thus obtained to a similar and convenient opacity with normal saline solution, sufficient formalin being added to make the concentration of the final emulsion 1 in 1000.

The agglutination tests were carried out in Dreyer's standardised tubes, 400 c.mm. of the serum dilution being mixed with 600 c.mm. of the formolised emulsion. The tubes were then placed in a water-bath at 50° to 55° C. Readings were made after the tubes had been at least two hours in the bath, and these were verified by a second reading made after the tubes had stood overnight at room temperature.

The results are shown in the following table:

TABLE I.

Strain.	Dilutions of serum. 1 in—								
	25	50	100	200	400	800	1600	3200	Control.
R.A.M.C. 2 . .	+	+	+	+	+	S. +	S. —	tr. —	—
R.A.M.C. 4 . .	+	+	+	+	+	S.	tr.	—	—
R.A.M.C. 5 . .	+	+	+	+	+	S.	tr. +	—	—
R.A.M.C. 6 . .	+	+	+	+	+	S. +	S.	tr. —	—
Galicia J.F. 7 .	+	+	+	+	+	S.	tr.	—	—
Pasteur Inst. 8 .	+	+	+	+	+	S. +	S. —	—	—
Pasteur Inst. M. 9 .	+	+	+	+	+	S. +	S.	—	—
Pasteur Inst. P. 10	+	+	+	+	+	S. +	S.	—	—
Italian 11 . . (homologous str.)	+	+	+	+	+	S.	tr.	—	—

The sign + indicates complete agglutination; — absence of agglutination; S. standard agglutination; tr. trace of agglutination.

Examination of these results shows that all the strains were agglutinated by very similar dilutions of the serum prepared by means of a vaccine made from the "Italian" strain.

Similar results were obtained when sera prepared by inoculating rabbits with vaccines made from the strains R.A.M.C. 2 and Galicia J.F. 7 were employed. The same technique was used. In Table II the results obtained with the latter serum are shown.

Strain Pasteur Inst. P. 10 was not tested.

TABLE II.

Strain.	Dilutions of serum. 1 in— 25	50	100	200	400	800	1600	3200	Control.
R.A.M.C. 2	+	+	+	+	+	+	+	S.	—
R.A.M.C. 4	+	+	+	+	+	+	S. +	tr.	—
R.A.M.C. 5	+	+	+	+	+	+	S.	—	—
R.A.M.C. 6	+	+	+	+	+	+	S.	—	—
Galicia J.F. 7 (homologous str.)	+	+	+	+	+	+	S.	—	—
Pasteur Inst. 8	+	+	+	+	+	+	S.	—	—
Pasteur Inst. M. 9	+	+	+	+	+	+	S.	—	—
Italian 11	+	+	+	+	+	+	+	S.	—

Although these results were so uniform it was thought necessary to confirm them by a series of tests in which the serum was tested for agglutinating power after it had been absorbed by thick emulsions of the various strains.

These tests were carried out as follows:

Agar slopes were planted with strain R.A.M.C. 2, Galicia J.F. 7, Pasteur Inst. M. 9, Pasteur Inst. P. 10 and Italian 11. After incubation overnight the resulting growths were washed off with 1 or 2 c.c. of salt solution to each agar slope. The opacity of these thick emulsions was then compared by making a series of dilutions in tubes of standardised bore; sufficient salt solution was then added, where necessary, to bring them all to, approximately, the same opacity.

Each emulsion was then divided into two portions, one of which was kept undiluted and the other was diluted 1 in 5 with salt solution. One c.c. of these undiluted emulsions and those diluted 1 in 5 respectively were then mixed with 1 c.c. of the serum, prepared by means of strain "Italian" 11, diluted 1 in 5. After being kept at room temperature for two hours the tubes containing the emulsions and serum were placed in the ice-chest overnight. As a control 2 c.c. of serum, diluted 1 in 5, was mixed with 2 c.c. of salt solution and the tube containing it treated in exactly the same manner.

The next morning all the tubes were centrifuged until the supernatant fluid was quite clear. This was then pipetted off and tested for agglutinating power in each case—(1) against the emulsion which had been used for absorbing the serum, (2) against an emulsion of strain "Italian" 11, which was the strain used in preparing the serum. These results were compared with those obtained with the control serum. The emulsions used in these tests were diluted peptone-water cultures containing 1 in 1000 of formalin.

In Table III the agglutinating powers of these absorbed and unabsorbed sera are given, the emulsions used in the agglutination tests being prepared from the vibrio used for absorbing the serum.

TABLE III.

Strain.	Treatment of serum.	Dilutions of serum used in agglutinating tests. 1 in—								Salt sol. control.
		50	100	200	400	800	1600	3200	6400	
Italian 11 (homologous str.)	Unabs. dil. 1/10	+	+	+	+	S.+	tr.+	tr.	—	—
Ditto	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.+	S.—	—	—	—	—	—
,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
R.A.M.C. 2	Unabs. dil. 1/10	+	+	+	+	S.	tr.	—	—	—
,,	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.+	S.—	—	—	—	—	—
,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Galicia J.F. 7	Unabs. dil. 1/10	+	+	+	+	S.	—	—	—	—
,, ,,	1/5 dil. abs. with emuls. dil. 1/5	+	+	+	S.	—	—	—	—	—
,, ,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Pasteur Inst. M.	Unabs. dil. 1/10	+	+	+	+	S.+	S.—	—	—	—
,, ,,	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.	—	—	—	—	—	—
,, ,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Pasteur Inst. P.	Unabs. dil. 1/10	+	+	+	S.+	S.—	—	—	—	—
,, ,,	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.	—	—	—	—	—	—
,, ,,	1/5 dil. abs. with undil. emuls.	S.	tr.	—	—	—	—	—	—	—

In Table IV the agglutinating powers of the same absorbed sera in respect of an emulsion of strain "Italian" 11 are shown. This strain of *V. choleræ* was the one used in the preparation of the serum.

TABLE IV.

Strain.	Treatment of serum.	Dilutions of serum used in agglutinating tests. 1 in—								Salt sol. control.
		50	100	200	400	800	1600	3200	6400	
Italian 11 (homologous str.)	Unabs. dil. 1/10	+	+	+	+	S.+	tr.+	tr.	—	—
Ditto	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.+	S.—	—	—	—	—	—
,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
R.A.M.C. 2	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.+	S.—	—	—	—	—	—
,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Galicia J.F. 7	1/5 dil. abs. with emuls. dil. 1/5	+	+	+	S.	—	—	—	—	—
,, ,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Pasteur Inst. M. 9	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.	tr.	—	—	—	—	—
,, ,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Pasteur Inst. P. 10	1/5 dil. abs. with emuls. dil. 1/5	+	+	+	S.	—	—	—	—	—
,, ,,	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—

The results obtained in these experiments conclusively confirm the results shown by the simple agglutination tests, namely, that, at any rate with the strains of *V. choleræ* which had been collected, there were no serological differences. As these strains were obtained from widely different sources it is probable that *V. choleræ* forms, serologically, a sharply defined group.

Further experiments showed that the serum prepared by injecting rabbits with the strains of *V. choleræ* "Italian" 11, R.A.M.C. 2 and Galicia J.F. 7 had no power of agglutinating suspensions made from cultures of *V. paracholeræ* A or B (Mackie) when the emulsions were made by diluting peptone-water cultures and adding formalin to bring the concentration to 1 in 1000.

In view of these results it was somewhat surprising that so many authors had given divergent opinions as to whether certain vibrios were true *V. choleræ*. The following experiments were therefore carried out, in which, instead of employing all the essential portions of Dreyer's technique* in making the agglutination tests, the bacterial emulsions were made by suspending in salt solution the vibrios grown on agar for eighteen to twenty hours at 37° C., the emulsions being then reduced to a similar opacity.

In Table V the results obtained with the serum "Italian" 11 are shown. The readings were made after the tubes had been kept at 50° to 55° C. for two hours in the water-bath.

TABLE V.

Strain.	Dilutions of serum. 1 in—						
	50	100	200	400	800	1600	Control.
Italian 11 (homologous str.)	+	+	+	+	S.	—	—
R.A.M.C. 2	+	+	S.+	S.—	—	—	—
R.A.M.C. 4	+	+	+	+	S.+	S.—	—
R.A.M.C. 5	+	+	+	+	S.—	tr.	—
R.A.M.C. 6	+	+	+	S.	—	—	—
Galicia J.F. 7	+	+	S.	tr.	—	—	—
Pasteur Inst. M. 9	+	+	+	+	S.	—	—
Pasteur Inst. 8	+	+	+	S.+	tr.	—	—
Pasteur Inst. P. 10	+	+	+	+	S.	—	—
V. parachol. A	+	+	+	S.+	tr.	—	—
V. parachol. B	tr.	+	+	+	S.	tr.	—

On examining these results it is at once seen that they are very irregular compared with those obtained with emulsions made from broth cultures, true *V. choleræ* strains being agglutinated to titres varying from 200 to nearly 1600, and the two strains of *V. paracholeræ* up to a titre of about 400 in the case of the A strain and 800 in the B strain. When, however, the same serum was employed together with an emulsion made from a diluted and formolised peptone-water culture neither the A nor B strain of *V. paracholeræ* was agglutinated by any dilution between 1 in 25 and 1 in 1600. On other occasions, however, especially when the time that the agglutination tubes were kept in the water-bath was considerably prolonged, emulsions made from agar cultures gave results approaching very closely those obtained with emulsions made from broth cultures.

---

* An account of Dreyer's technique will be found in the Medical Research Council's Special Report Series, No. 51, London, 1920 (H.M. Stationery Office), p. 136.

These results emphasise the importance of using in all similar investigations emulsions made from broth cultures, as recommended by Dreyer, which have been diluted to a suitable opacity with salt solution and formolised to a concentration of 1 in 1000.

Having found that *V. choleræ* probably formed a compact serological group, cultures of a number of other vibrios were collected and tested to ascertain if any of them were agglutinated by serum made from a strain of *V. choleræ*.

Besides the two strains of *V. paracholeræ* A and B, cultures of *V. nasik* and the El Tor vibrios A, 4, 5, 10 and 35 were collected. These cultures were frequently subcultured both on solid and liquid media, and then emulsions were made by growing the organisms in broth for eighteen to twenty-four hours at 37° C., diluting to a convenient and equal opacity and adding sufficient formalin to make the final concentration equal to 1 in 1000.

The first organism to be tested was *V. nasik*. The results of the agglutination tests with the three sera prepared from the strains of *V. choleræ* "Italian" 11, R.A.M.C. 2 and Galicia J.F. 7 are shown in the following table:

TABLE VI.

Strain used to prepare serum.	Strain used to make emuls.	Dilutions of serum. 1 in—							
		50	100	200	400	800	1600	3200	Control.
Italian 11	Italian 11	+	+	+	+	+	S.	—	—
,,	*V. nasik*	—	—	—	—	—	—	—	—
R.A.M.C. 2	Italian 11	+	+	+	+	+	+	S.+	—
,,	*V. nasik*	—	—	—	—	—	—	—	—
Galicia J.F. 7	Italian 11	+	+	+	+	+	+	S.+	—
,, ,,	*V. nasik*	—	—	—	—	—	—	—	—

These results show that emulsions of *V. nasik* are not agglutinated by the serum made from any of the strains of *V. choleræ*.

The next experiments were carried out with the El Tor vibrios and with *V. paracholeræ* A and B. In order to assist in subdividing these organisms into serological groups, a serum was prepared by inoculating a rabbit with a suitable dose of vaccine prepared from *V. paracholeræ* A.

Emulsions of *V. paracholeræ* A, even when made by diluting and formolising broth or peptone-water cultures, were *now* found to be agglutinated by sera prepared from strains of *V. choleræ* to a titre somewhat higher than similar emulsions of the homologous vibrio.

This property, which, as has been mentioned above, was completely absent when the culture was first obtained, only gradually developed after repeated subcultures had been made on fluid and solid media.

Once the property had been developed it remained constant, and the specificity, which was present when the culture was first obtained, was not regained either by repeated daily subcultures in fluid media or the passage by intraperitoneal and subcutaneous injection through a series of sixteen guinea-pigs, a proceeding which completely restored the virulence.

The first series of agglutination tests were carried out with a cholera agglutinating serum prepared by means of the strain *V. choleræ* "Italian" 11.

Table VII shows the results obtained with various organisms.

TABLE VII.

Strain.	Dilutions of serum. 1 in—										
	25	50	100	200	400	800	1600	3200	6400	12800	Control.
V. parachol. A	+	+	+	+	+	+	+	+	S.+	tr.+	—
V. parachol. B	—	—	—	—	—	—	—	—	—	—	—
V. El Tor A.	+	+	+	+	+	+	S.	0	0	0	—
V. El Tor 4	+	+	+	+	+	+	+	S.—	tr.—	0	—
V. El Tor 5	+	+	+	+	+	+	S.	0	0	0	—
V. El Tor 10	—	+	+	+	+	+	+	0	0	0	—
V. El Tor 35	—	—	—	—	—	—	—	0	0	0	—
V. chol. Italian 11 Control	0	0	+	+	+	S.+	tr.	—	—	0	—

On examining this table it is seen that emulsions of El Tor vibrios A, 4 and 5 were agglutinated to a titre approximately the same as the homologous vibrio, while emulsions of *V. paracholeræ* A and the El Tor vibrio 10 were agglutinated to a titre considerably higher. The emulsions of *V. paracholeræ* B and the El Tor vibrio 35 were not agglutinated by any of the dilutions of the serum employed.

Absorption experiments were therefore carried out to ascertain if the El Tor vibrios A, 4, 5 and 10 belonged to the *V. choleræ* or to the *V. paracholeræ* A group, and also to ascertain if emulsions of *V. paracholeræ* A had acquired the property of absorbing the agglutinating power from a cholera agglutinating serum. In the following tables are shown the results obtained. The undiluted emulsions used in absorbing the serum contained 100,000 million vibrios per c.c., and equal volumes of the absorbing emulsion were added to an equal volume of the serum diluted 1 in 10.

In Table VIII the serum was prepared with the strain of *V. choleræ* "Italian" 11.

TABLE VIII.

Serum 1/10 dil. prepared from str. Italian treated with—	Strain used in the preparation of the emulsion used in the agglutination tests.	Dilutions of serum for agglutination test. 1 in—							
		100	200	400	800	1600	3200	6400	Control.
Salt sol. control	Italian 11	+	+	+	S.+	tr.	—	—	—
Abs. with undil. emuls. of *V. paracholera* A.	Italian 11	+	+	+	S.—	—	—	—	—
Abs. with emuls. of *V. parachol.* A dil. 1/5	Italian 11	+	+	+	S.+	tr.	—	—	—
Abs. with emuls. of *V. parachol.* A dil. 1/25	Italian 11	+	+	+	+	S.+	tr.	—	—
Abs. with undil. emuls. of V. El Tor A.	Italian 11	—	—	—	—	—	—	—	—
Abs. with emuls. of V. El Tor A dil. 1/5	Italian 11	—	—	—	—	—	—	—	—
Salt sol. control	V. El Tor A.	+	+	+	S.	—	—	—	—
Abs. with undil. emuls. of V. El Tor 5	Italian 11	—	—	—	—	—	—	—	—
Abs. with emuls. of V. El Tor 5 dil. 1/5	Italian 11	—	—	—	—	—	—	—	—
Salt sol. control	V. El Tor 5	+	+	+	S.	—	—	—	—
Abs. with undil. emuls. of V. El Tor 4	Italian 11	—	—	—	—	—	—	—	—
Salt sol. control	V. El Tor 4	+	+	+	S.+	—	—	—	—

On examining the results shown in this table it is seen that vibrios El Tor A, 4 and 5 absorbed from the cholera agglutinating serum the power to agglutinate emulsions of the *V. choleræ* "Italian" 11 which had been used to prepare the serum. These strains of the El Tor vibrios must, therefore, be considered to be strains of true *V. choleræ.* As regards *V. paracholeræ* A, although emulsions of this organism were found to have acquired the property of being constantly agglutinated by serum prepared with strains of *V. choleræ*, even a volume of the serum diluted 1 in 10 absorbed with an equal volume of emulsion containing 100,000 million vibrios per c.c. was only reduced in the agglutinating power by 30 per cent. When the absorbing emulsion contained 20,000 million vibrios per c.c. there was no reduction in the agglutinating power of the serum, and when the absorbing emulsion contained 4000 million vibrios per c.c. it was found actually to have acquired an increased agglutinating power towards emulsions made from the homologous strain of *V. choleræ.* (This last observation illustrates a somewhat uncommon phenomenon occasionally noticed when sera are absorbed with an organism belonging to a different species to that used in preparing the serum.) These observations show that although *V. paracholeræ* A had become agglutinable with sera prepared from *V. choleræ* this was not specific agglutination. Further confirmation of the difference in agglutinating properties of *V. choleræ* and *V. paracholeræ* A is also shown in Table IX, where it is seen that a serum prepared from *V. paracholeræ* A, although agglutinating emulsions of this organism in dilutions up to 1 in 3200, failed to agglutinate emulsions of *V. choleræ* even in dilutions of 1 in 100.

TABLE IX.

Serum dil. 1/10 prepared from *V. parachol.* A treated with—	Strain used in preparation of the emulsion used in the agglutination tests.	Dilutions of serum for agglutination tests. 1 in— 100	200	400	800	1600	3200	6400	Control.
Salt sol. control	*V. parachol.* A	+	+	+	+	S.+	S.	—	—
Salt sol. control	V. El Tor 10	+	+	+	+	+	S.+	—	—
Abs. with undil. emuls. of V. El Tor 10	*V. parachol.* A	—	—	—	—	—	—	—	—
Abs. with emuls. of V. El Tor 10 dil. 1/5	*V. parachol.* A	tr.	—	—	—	—	—	—	—
Abs. with emuls. of V. El Tor 35 undil.	*V. parachol.* A	+	+	+	+	+	S.	—	—
Abs. with emuls. of V. El Tor 35 dil. 1/5	*V. parachol.* A	+	+	+	+	+	S.	—	—
No treatment	*V. chol.* Ital.	—	—	—	—	—	—	—	—

Table IX also shows the results of absorbing a serum made by means of *V. paracholeræ* A in respect of its agglutinating power with emulsions of the El Tor vibrios 10 and 35. They indicate that the El Tor vibrio 10 completely absorbed the agglutinating power from the serum in respect of emulsions of *V. paracholeræ* A, so that El Tor vibrio 10 must be considered identical with *V. paracholeræ* A.

The El Tor vibrio 35, however, showed no absorption and must therefore be considered distinct from the *V. paracholeræ* A group. Unfortunately no serum had been prepared from *V. paracholeræ* B, so it was not possible to ascertain if this organism was identical with that vibrio.

SUMMARY.

The results obtained show that:

(1) All the strains of *V. choleræ* investigated belonged to one serological race, and as these strains came from widely different sources it is probable that there is only one serological race of *V. choleræ*.

(2) The advantage of using emulsions made by formolising broth cultures as recommended by Dreyer when comparing the agglutinating properties of a series of strains of the same organism is sharply brought out.

(3) After continued subculture on artificial media emulsions of *V. paracholeræ* A acquired the property of being agglutinated by cholera-agglutinating sera, but these emulsions had no power of absorbing the agglutinins for emulsions of the homologous vibrio. This property was not lost either by repeated daily subculture in fluid media or by passage through a series of guinea-pigs sufficiently numerous completely to restore the virulence of the organism.

(4) Of the El Tor vibrios, the strains known as A, 4 and 5 behaved both in their agglutinating and absorbing properties as true *V. choleræ* and should therefore be considered as belonging to this species. Strain 10 behaved in an exactly similar manner to *V. paracholeræ* A, being agglutinated by an anti-serum prepared from this organism and absorbing the specific agglutinins from the *V. paracholeræ* A anti-serum, and must therefore be considered as belonging to this species. *V. nasik*, *V. paracholeræ* B and the El Tor vibrio 35 had no serological relation with either *V. choleræ* or *V. paracholeræ* A and could not be classified.

THE GASTRIC AND RESPIRATORY RESPONSE TO MEALS.

T. IZOD BENNETT,

Beit Memorial Fellow for Medical Research,

AND

E. C. DODDS.

From the Physiological Department and the Bland-Sutton Institute of Pathology, Middlesex Hospital, London.

Received for publication February 10th, 1921.

In previous communications ([1] [2]) we have independently called attention to (*a*) the various types of gastric secretion and motility found in normal men (by T. I. B.), and (*b*) the alterations in the tension of alveolar CO_2 which follow the ingestion of a meal (by E. C. D.). A full account of the first subject will be found in an article written in conjunction with J. A. Ryle in the current number of *Guy's Hospital Reports.*([3])

In the present article we bring forward the results of work done in collaboration, in which the responses of a series of individuals to both tests may be compared; we believe that by this means we have produced corroboration of one another's findings, and that examination of the parallel results may throw some light on problems connected with gastro-intestinal secretion.

The subjects from whom the present results were obtained were, in two respects, selected ones:

(1) They were all men in good health, without symptoms or previous history of gastro-intestinal disease.

(2) Some of them were chosen for alveolar air examination because their gastric analyses had shown them to be variants from the more usual type.

For indications as to what proportion of such cases may be found among normal men, the reader is referred to the article by Bennett and Ryle mentioned above.

TECHNIQUE.

A. *Gastric Analysis.*—The method used was the fractional one introduced by Rehfuss ([4, 5]), the exact technique following closely that employed by Crohn and Reiss.([6])

The test-meal consists of a pint of thin oatmeal gruel, which gives a gastric response quantitatively slighter than that obtained with a bulkier meal of higher protein content. Ryle's modification of the Einhorn duodenal tube(7) is swallowed by the fasting subject, and after all the "resting-juice" has been withdrawn, the meal is swallowed and samples are withdrawn every quarter-hour until the stomach is empty.

These samples are then analysed, the percentage of free hydrochloric acid and the total acidity being estimated in each case by titration with decinormal alkali, using dimethylamidoazobenzol and phenolphthalein as indicators. The disappearance of the meal from the stomach is estimated by noting the last sample in which starch, as tested for by iodine, is present.

B. *Estimation of alveolar carbon dioxide tension.*—It will be well to refer at this point very briefly to the results of an earlier investigation.(2) In a series of twelve normal persons the alveolar carbon dioxide pressure was found to show the following changes after a meal: (1) A rise of from 2 to 6 mm. within the first half or three-quarters of an hour; (2) a subsequent fall of about the same amount (2–6 mm.) below the original level; (3) a return to this level. In a man from whom the greater part of the stomach had been removed, the rise after a meal was very small in amount (0·4 to 0·8 mm.), while the subsequent changes were similar to those seen in a normal person. It seemed probable that the rise was associated with the secretion of gastric juice, and the subsequent fall with the later processes of digestion.

In the later observations on CO_2 tension which are dealt with in the present paper it seemed best to adhere to that form of meal with which previous experience had been gained, although this is different from the test-meal of gruel. After the subject had rested for half an hour samples of alveolar air were taken shortly before lunch, which consisted of beef, potatoes, bread and cheese; after the meal samples were taken every quarter or half-hour until the tension remained approximately constant, near the original level, in two successive samples. It was, of course, necessary to be sure that the changes in alveolar carbon-dioxide tension following upon breakfast were not still in progress: the subjects were instructed to take breakfast not later than 8 a.m., and the duplicate samples of alveolar air taken before lunch were separated by a quarter of an hour's interval so that the

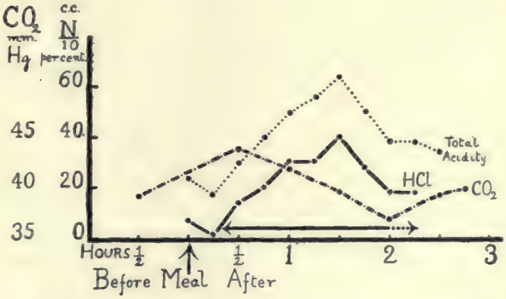


FIG. 1.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of usual type (Case 1). The arrow in this and subsequent figures indicates the emptying time of the stomach, as shown by the duration of a positive iodine test for starch. A dotted portion of the arrow indicates a faint trace of starch only.

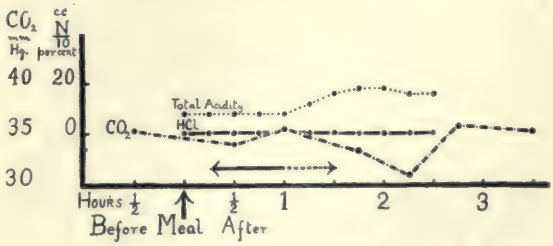


FIG. 2.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of achlorhydric type (Case 10).

meal could be deferred if any distinct difference between these two samples were found. All samples were taken by the Haldane-Priestley method, each point on the curve being the mean of two samples—an expiratory and an inspiratory.

RESULTS.

Each of the four normal types previously described (1) was examined.

(1) *The usual type* (Fig. 1, Case 1).—The curve of gastric HCl percentage here shows a steady rise after the test-meal, reaching a height between 20 and 50 c.c. of N/10 per cent., and then falling as the regurgitation of alkaline duodenal contents occurs at the end of gastric digestion.

The tension of alveolar CO_2 shows the rise followed by a fall, which has already been observed and ascribed to gastric secretion and a later secretion in lower portions of the alimentary tract.

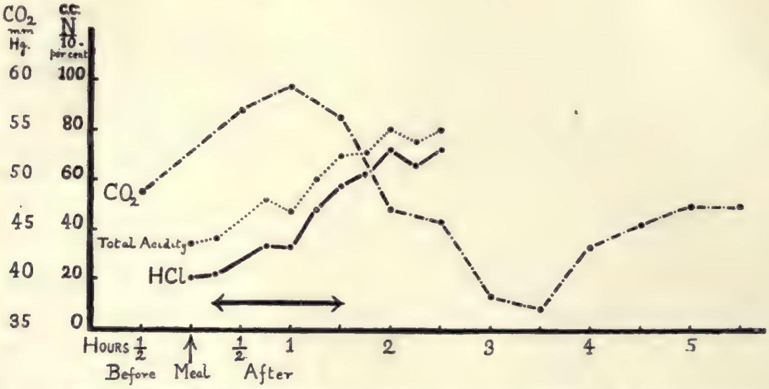


FIG. 3.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of "hyperchlorhydric" or "hypersecretory" type (Case 11).

We have observed curves in all ways similar to these in eight cases examined by the double method.

(2) *The type with low gastric acidity or achlorhydria* (Fig. 2, Case 10).—The example illustrated is from a man, apparently quite healthy, who at no time shows any free HCl to be present in his stomach. Comment on the gastric analysis is superfluous; the curve of alveolar CO_2 tension is strikingly similar to those obtained from a patient who had had a complete gastrectomy, which have been published elsewhere. (2)

(3) *The type with gastric hypersecretion or hyperchlorhydria* (Figs. 3 and 4).—The first example illustrated (Fig. 3) is from a healthy man (Case 11), who shows hyperchlorhydria in rather an extreme degree. At the end of an hour the free HCl reaches 50 c.c. N/10 per cent., whereas the average in a large series is not higher than about 30 c.c.; more striking still is the fact that after this point the acidity continues to rise, probably being helped by the absence of the duodenal regurgitation which occurs in the majority of normal persons.

The alveolar CO_2 tension in the same subject shows a remarkable range, rising no less than 10 mm. after the meal and then falling to a similar extent before returning to its original level.

The second example (Fig. 4, Case 12) of this type is equally remarkable; the gastric analysis does not at first glance reveal very remarkable figures, as the acidity does not rise much beyond 50 c.c. of N/10 per cent., but there is the same absence of fall at the end of the curve that was present in the previous subject. It was observed, however, that in this subject 140 c.c. of juice were present in the resting stomach, whereas the usual figure is about 40 c.c.; and at the end of three hours a little of the test-meal was still present and no less than 460 c.c. of gastric contents were removed. In fact the 50 c.c. of N/10 per cent. present in the stomach really represented an enormous secretion of gastric juice. We were therefore not surprised to find that this individual gives a rise of 15 mm. in alveolar CO_2 tension after a meal, which is greater than that of our previous subject No. 11. Further comment on these findings will be found in a later section of this paper.

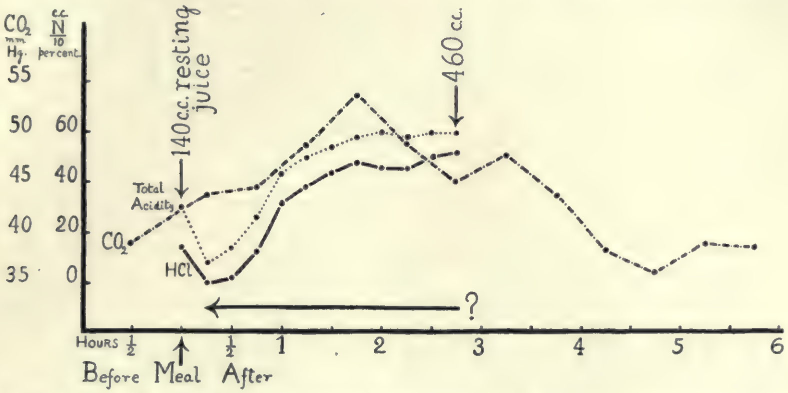


FIG. 4.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of "hyper-secretory" or "hyperchlorhydric" type (Case 12).

(4) *The rapid type* (Fig. 5, Case 7).—The subjects of whom an example is here shown have a normal gastric acidity but a remarkably rapid gastric cycle; exactly parallel results have been obtained in the alveolar CO_2 estimations (Cases 7 and 8). In many of these subjects samples must be taken at very frequent intervals if the variations are to be observed. The stomach has emptied itself after ¾ hour, whereas in the more usual type this process takes from 1½ to 2½ hours.

These three less usual types are shown together in Fig. 6.

In Table I the figures are given from twelve of the cases we have investigated together; they include only a certain propor-

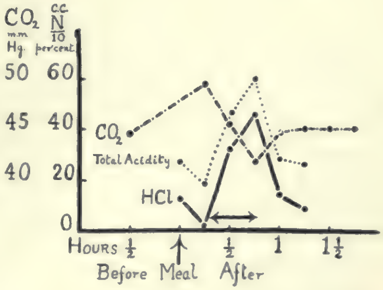


FIG. 5.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of "rapid" type (Case 7).

tion of our results, and are published merely to illustrate the type of results of which the curves in the text are examples.

In Table II will be found corresponding figures (*a*) to convert c.c. N/10 per cent. into the true percentage of HCl, and (*b*) to convert tension of alveolar CO_2 in mm. Hg. into actual percentage of CO_2, this latter table being applicable when the atmospheric pressure for the day is 760 mm. Hg.

COMMENTARY.

Further investigation of these cases is in progress, and we hope at a later date to publish, in conjunction with E. L. Kennaway, the results of urinary and blood examinations in the same series. Until the complete figures are before us we prefer to reserve full discussion of our findings. To three things, however, we wish now to draw attention:

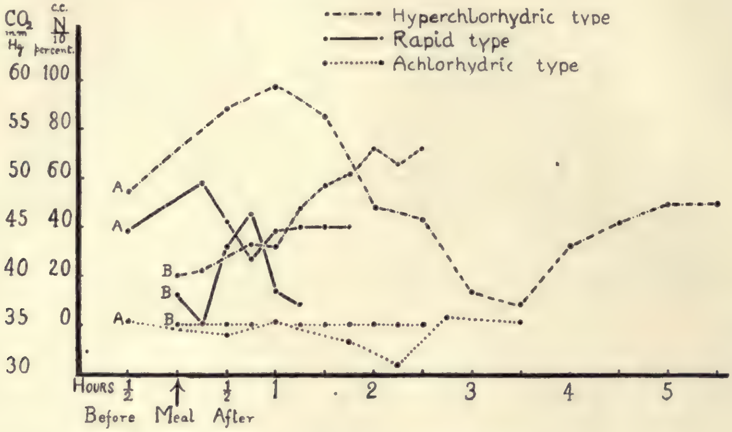


Fig. 6.—To contrast the curves of the three less usual types:
Example shown of hyperchlorhydric type is Case 11.
" " rapid " " 7.
" " achlorhydric " " 10.
Curve A: Alveolar CO_2, mm. Hg. Curve B: Free HCl, c.c. $\frac{N}{10}$ per cent.

(1) To the evidence in Fig. 2 of normal secretion below the pylorus, even in the absence of gastric HCl. In a recent article on achylia gastrica,(8) one of us summarised the functions of the gastric HCl as follows: "It is a useful but merely accessory agent for protein digestion, a useful but accessory agent for controlling the action of the gastric sphincters, an important but not essential means of diminishing bacterial growth in the intestines."

The immunity from symptoms of many persons with achlorhydria has long been observed; the curve of alveolar CO_2 tension in these cases supports the belief that the secretions in lower portions of the alimentary canal can proceed normally in the absence of any excitation due to hydrochloric acid.

(2) To the differences in alveolar CO_2 tension in different subjects during the "resting" period of digestion. In Fig. 6 will be found plotted together the results from several different types; it will be observed that those whose

TABLE I

		BEFORE MEAL		AFTER MEAL																		
CASE Nº		½ HOUR BEFORE MEAL	JUST 0 BEFORE MEAL	¼ HO	½ URS	¾	1	1¼	1½	1¾	2	2¼	2½	2¾	3	3¼	3½	4	4½	5	5½	
1.	A		7	0	14	20	29	30	40	28	18	18	18									AVERAGE TYPE VIDE FIG. 1.
	B		24	18	30	40	51	56	64	50	38	40	42									
	C	39·2			43·8		41·9		39·6		36·9		39·2	39·2								
2.	A		0	0	4	16	32	40														AVERAGE TYPE RATHER RAPID
	B		10	8	20	34	46	54														
	C	39·8			45·3		36·7		34·5		40·4		41·2									
3.	A		26	0	4	10	22	20	22	2	16	10	12									AVERAGE TYPE
	B		38	10	16	24	34	38	40	14	26	26	22									
	C	38·4			40·5		38·6		36·3		37·8		38·6									
4.	A		14	2	18	28	18	24	22	24	22	14	10									AVERAGE TYPE
	B		26	20	34	44	36	42	40	42	40	24	22									
	C	38·9			44·9		41·3		37·8		34·7			38·9	38·9							
5	A		0	0	0	12	16	16	20	14	10	15	20									AVERAGE TYPE
	B		5	6	9	25	29	33	42	34	27	36	30									
	C	39·2		42·2	42·6		42·6		42·2		40·3		33·9		39·9	39·6						
6.	A		0	0	0	9	12	16	18	18	18											AVERAGE TYPE
	B		16	6	11	29	34	36	36	33	33											
	C	40·1		42·4	41·6		43·2		40·1		38·2		39·9	40·4								
7.	A		12	0	32	46	12															RAPID TYPE VIDE FIG. 5
	B		34	16	44	64	25															
	C	44·7		49·5	45·5	41·7	44·7	45·1	45·1	45·1												
8.	A		0	0	6	20	30															RAPID TYPE
	B		11	14	24	31	44															
	C	39·1		42·8	38·6	32·5	37·5	38·2	39·1	39·1												
9.	A		0	0	0	0	0	0	0													ACHLORHYDRIC TYPE
	B		6	6	6	8	8	6	6													
	C	43·6		44	43·6		38·2	38·2	39·3	44·3	43·5		43·6									
10	A		0	0	0	0	0	0	0	0	0	0	0									ACHLORHYDRIC TYPE VIDE FIG. 2
	B		8	8	8	8	8	12	16	18	18	16	16									
	C	35·3			33·9		35·3			33·2		30·9		35·7	35·3							
11	A		20	22	?	33	32	48	57	62	72	66	72									HYPERSECRETION TYPE VIDE FIG. 3
	B		34	36	?	53	46	60	67	69	80	75	80			SEPARATED BY ½ HR INTERVALS						
	C	48·6			57·0		59·3		56·2		47·1		45·9		38·4		37·1	43·2	45·5	47·4	47·4	
12	A		14	0	2	12	32	38	44	48	46	46	50	52								HYPERSECRETION TYPE VIDE FIG. 4
	B		30	8	14	26	44	50	54	58	60	58	60	60		SEPARATED BY ½ HR INTERVALS						
	C	38·8		43·6		44·5		48·7		53·6		49·1		45·3		47·9	44·1	38·4	36·1	39·2	38·8	

A = FREE HCl EXPRESSED IN CC $\frac{N}{10}$ PER CENT.
B = TOTAL ACIDITY IN CC $\frac{N}{10}$ PER CENT.
C = ALVEOLAR CO$_2$ TENSION IN MM.

percentage of HCl later reaches a high figure show a high alveolar CO_2 tension, even before gastric digestion has begun; the converse is seen in cases of achlorhydria. The explanation of this is that gastric secretion, like gastric peristalsis, is a more continuous process than has been usually believed, the cycle of secretion that follows a meal being merely the accentuation of a process that continues in a much slighter degree at other times; much light has been thrown on this mechanism by the recent work of Carlson.(9)

(3) To the necessity for revising existing views as to the function of the respiratory centre.

At the present stage there does not seem to us to be any purpose in considering how far subjects such as the two with hyperchlorhydria, whose alveolar CO_2 tension ranges over as much as 20 mm. in the course of a few hours, are to be regarded as normal or pathological. We believe it to be a new observation that any respiratory centre, normal or abnormal, allows rapid changes of such magnitude to occur. It is evident that the centre does not maintain the CO_2 tension at a constant level; it seems rather to produce such variations in this tension as are appropriate to maintain some other factor constant, and in some persons these variations have to be very considerable. If the H-ion concentration of the blood is to be kept approximately constant these large fluctuations in alveolar CO_2 tension are bound to occur. For instance, when free hydrochloric acid is formed from the neutral body fluids and secreted into the stomach, an equivalent quantity of base must be left behind which will disturb the proportion $H_2CO_3/NaHCO_3$ upon which the reaction of the blood depends. It is then necessary to restore the balance by retaining carbon dioxide; if the amount of gastric acid secreted has been considerable as in our subjects 11 and 12 the lung ventilation may have to be lessened till the CO_2 tension has risen by as much as 10 mm. (Figs. 3 and 4).

TABLE II

CONVERSION OF $\frac{N}{10}$ NaOH PER CENT. INTO PERCENTAGE OF HCl.

CC $\frac{N}{10}$ NaOH	10	20	30	40	50	60	70	80	90
HCl PER CENT	·036	·073	·109	·146	·182	·219	·255	·292	·327

CONVERSION OF ALVEOLAR CO_2 TENSION AT 760 MM OF MERCURY INTO CO_2 PERCENTAGE.

CO_2 PER CENT	4	4·5	5	5·5	6	6·5	7
CO_2 TENSION	30·4	34·2	38·0	41·8	45·6	49·4	53·2

The respiratory centre, then, does not maintain a constant alveolar CO_2 tension. It may appear to do so if observations are made when no active digestion is in progress, because CO_2 is at these times by far the most abundant substance of acid or alkaline reaction which is entering or leaving the blood, and the maintenance of its tension at a constant level is then just what is required, in conjunction with the activity of the kidney, to preserve the ratio $H_2CO_3/NaHCO_3$ at its proper amount. That the alveolar CO_2 tension is preserved constant no longer than is suitable for the needs of the body has, of course, long been known in the case of acclimatisation to high altitudes; the observations brought forward in this paper show further that considerable variations occur as the result of an ordinary meal.

SUMMARY.

(1) Studied by means of gastric analysis, and by estimation of alveolar CO_2 tension, normal men are found to fall into four distinct classes.

(2) The results obtained by the two methods are found to confirm one another.

(3) Cases showing achlorhydria during gastric analysis show a curve of alveolar CO_2 tension similar to that obtained in patients following gastrectomy.

(4) Such cases show a fall in alveolar CO_2 tension at a later period, indicating normal secretion during the later processes in digestion.

(5) Cases of hyperchlorhydria in healthy men have sometimes been found to show changes in alveolar CO_2 tension following meals which exceed what have previously been considered physiological limits.

(6) Some normal cases occur in whom the processes of upper alimentary digestion are so rapid as to be only demonstrable by observations taken at very short intervals.

(7) Cases which yield a high gastric acidity during digestion are usually found to show a relatively high tension of alveolar CO_2 even during the "resting" phases of the stomach; in cases of achlorhydria the reverse is seen.

We wish to acknowledge our indebtedness to all those gentlemen, students of Guy's and the Middlesex Hospitals, who have so willingly given their time, often at great inconvenience, to serving as subjects for these experiments.

We also wish to acknowledge the constant and critical help of Dr. E. L. Kennaway.

The respiratory observations made in this work form part of a report to the Medical Research Council.

REFERENCES.

1. Bennett, T. I.—"Observations made by means of the Fractional Method of Gastric Analysis," *J. Physiol.*, 1920, **54**, xlv.
2. Dodds, E. C.—"Variations in Alveolar Carbon Dioxide Pressure in Relation to Meals," *J. Physiol.*, 1921, **54**, 342.
3. Bennett, T. I., and Ryle, J. A.—"Studies in Gastric Secretion," *Guy's Hosp. Rep.*, 1921, **71** (in the press).
4. Rehfuss, M. E.—"A New Method of Gastric Testing," *Amer. J. Med. Sci.*, 1914, **147**, 848.
5. Rehfuss, M. E., Bergeim, O., and Hawk, P.—"Gastro-intestinal Studies II," *J. Amer. Med. Assoc.*, 1914, **63**, 909.
6. Crohn, B. B., and Reiss, J.—"Studies in the Fractional Estimation of Stomach Contents," *Amer. J. Med. Sci.*, 1917, **154**, 857.
7. Ryle, J. A.—"Studies in Gastric Secretion," *Guy's Hosp. Rep.*, 1921, **71**, 43.
8. Bennett, T. I.—"Achylia Gastrica," *Lancet*, 1920, **2**, 1196.
9. Carlson, A. J.—'The Control of Hunger in Health and Disease,' Chicago, 1916.

AN INQUIRY INTO THE DISTRIBUTION OF THE BLOOD GROUPS IN PATIENTS SUFFERING FROM "MALIGNANT DISEASE."

W. ALEXANDER, M.B., Ch.B. St. And.

From the Bacteriological Department, University of St. Andrews, University College, Dundee.

Received for publication January 25th, 1921.

The following is a report of an investigation which is being carried out in the bacteriological laboratories of the University of St. Andrews, the object of which is to determine the distribution of the blood groups in individuals known to be suffering from "malignant disease."

The number of cases so far dealt with—fifty—is not sufficiently large to justify any definite conclusions being drawn, and all that is intended is to call attention to one or two features which have presented themselves in the course of the inquiry.

We know that blood-grouping follows Mendelian laws, but we are ignorant of what really constitutes "group": and we are also ignorant of the mechanism of hæmagglutination. Again, we do not know all the factors that determine the occurrence of malignant disease, but there is more than a suggestion that a hereditary factor, as distinct from inherited infection, plays some part in its causation. In view thereof, it was decided to carry out the investigation herein described.

Figures relating to the blood groups of persons suffering from tuberculosis, syphilis and tetanus—infective diseases of known ætiology, and susceptible to investigation by immunological methods—are included in the series. These serve the double purpose of controlling the results obtained in cases of malignant disease, and of showing that susceptibility to the infective maladies is in no way related to the blood groupings.

Should it ultimately be proved that individuals belonging to certain of the blood groups are more susceptible to malignant neoplasms than those belonging to others, a stimulus is given to inquire into the innate qualities that may be associated with susceptibility to malignant disease.

Under the term "Malignant disease" we have included cases of carcinoma, sarcoma, rodent ulcer and leukæmia, this last condition being regarded provisionally as a sarcoma of the hæmopoietic system. In the majority of cases of the present series the clinical evidence has been verified by post-mortem and microscopical examination, and all cases in which the Wassermann reaction was positive have been excluded from the series.

The routine method was to test the patient's corpuscles against sera known to belong to Groups II and III, and the converse reaction, *i. e.* testing the serum of the patient under examination against corpuscles of known type, was only performed where any doubt as to the blood group arose, or as a confirmatory test in those cases where no agglutination was observed—that is, in all cases of Group IV. In selecting sera for test purposes, it is important that only those be employed which give specific results when tested by examination of a sampling of a general healthy population, and which give, when so tested, an approximately correct statistical result.

As a rule there was no difficulty in determining when agglutination had occurred, but it was considered advisable to set a time limit, and if agglutination was not obvious with one or other within 60 seconds when Group II and Group III sera were used, the patient was regarded provisionally as belonging to Group IV. Sometimes there was a tendency to rouleaux formation, and this may be mistaken for a positive result, and clotting may also produce an appearance simulating agglutination, but by the adoption of a time limit this latter danger may be eliminated.

TABLE I.—*Showing Distribution of Blood Groups in Fifty Cases of Malignant Disease.*

No.	Sex.	Age.	Disease.	Group.
1	M.	58	Epithelioma tongue	I.
2	M.	43	Carcinoma head of pancreas	I.
3	M.	39	Carcinoma head of pancreas	I.
4	F.	54	Carcinoma cervix uteri	I.
5	F.	59	Carcinoma breast	I.
6	F.	80	Rodent ulcer nose	I.
7	M.	60	Epithelioma bladder	II.
8	F.	37	Carcinoma sigmoid	II.
9	M.	64	Rodent ulcer nose	II.
10	M.	62	Carcinoma rectum	II.
11	M.	60	Carcinoma colon	II.
12	F.	52	Scirrhus breast	II.
13	F.	56	Carcinoma stomach	II.
14	M.	68	Sarcoma testicle	II.
15	F.	44	Malignant ovarian cyst ; carcinoma stomach	II.
16	F.	58	Carcinoma sigmoid	II.
17	F.	62	Carcinoma rectum	II.
18	M.	60	Carcinoma sigmoid	II.
19	M.	57	Carcinoma pylorus	II.
20	M.	24	Sore on forearm, flat-celled epithelioma	II.

No.	Sex.	Age.	Disease.	Group.
21	M.	49	Carcinoma colon	III.
22	M.	67	Carcinoma colon	III.
23	M.	40	Carcinoma jaw	III.
24	M.	60	Carcinoma pylorus	III.
25	F.	70	Carcinoma stomach	III.
26	M.	83	Epithelioma tongue	III.
27	F.	46	Carcinoma head of pancreas	III.
28	M.	46	Carcinoma larynx	III.
29	F.	22	Myeloid leukæmia	III.
30	F.	20	Malignant tumour thyroid	III.
31	M.	47	Carcinoma stomach	III.
32	F.	44	Cylinder - celled epithelioma, sacral region	III.
33	F.	84	Rodent ulcer	III.
34	F.	38	Carcinoma œsophagus	III.
35	F.	57	Carcinoma cervix uteri	III.
36	F.	62	Carcinoma cervix uteri	III.
37	M.	54	Carcinoma colon	IV.
38	M.	49	Carcinoma rectum	IV.
39	F.	66	Carcinoma stomach	IV.
40	F.	72	Carcinoma rectum	IV.
41	M.	66	Rodent ulcer nose	IV.
42	F.	20	Myeloid leukæmia	IV.
43	M.	50	Flat-celled epithelioma nose	IV.
44	F.	24	Carcinoma rectum	IV.
45	F.	51	Scirrhus breast	IV.
46	F.	58	Carcinoma rectum	IV.
47	M.	55	Carcinoma stomach	IV.
48	M.	66	Carcinoma jaw	IV.
49	M.	40	Sarcoma testicle	IV.
50	F.	45	Carcinoma pylorus	IV.

In Table II these results are compared with those obtained in examining a similar number of specimens from (1) a normal population; (2) cases of pulmonary tuberculosis; (3) cases of active syphilis; (4) cases of tetanus. For information concerning these last I am indebted to Dr. W. J. Tulloch.

TABLE II.—*Showing a Comparison between the Incidence of the Blood Groups in Malignant Disease with that in Other Conditions.*

	Per cent. in 50 normal.	Per cent. in 50 cases of tuberculosis.	Per cent. in 50 cases of active syphilis.	Per cent. in 25 cases of tetanus.	Per cent. in 50 cases of malignant neoplasm.
Group I	4	4	4	4	12
Group II	38	34	36	32	28
Group III	12	12	12	16	32
Group IV	46	50	48	48	28

From the above figures it appears that individuals belonging to all four blood groups are liable to suffer from malignant disease. It would seem, however, that Groups I and III are peculiarly susceptible to the various forms of malignant new growth, while Group IV is more stable or resistant in this respect. The figures are insufficient to permit of any suggestion being offered concerning the susceptibility of Group II.

These findings are borne out from the clinical aspect of the cases, for speaking generally, carcinoma or sarcoma occurring in Groups I or III of the present series proved to be of a severe kind and the course rapid; on the other hand, in the case of Groups II and IV, especially Group IV, the tumour was usually of low malignancy and the duration of the disease long.

These results are published in the hope that workers in other parts may be tempted to carry out the blood grouping of cases of malignant disease so that an adequate statistic may soon be available.

In conclusion, I should like to express my indebtedness to Dr. Tulloch for many excellent suggestions and assistance, and also to Prof. L. R. Sutherland for supplying me with pathological reports on the cases.

To Dr. John Taylor and Dr. G. R. Ross, I owe a special debt of gratitude as they were the willing sources of my type sera. And lastly I have to thank the Superintendent and Consultant Staff of the Royal Infirmary, The Royal Victoria Hospital and King's Cross Hospital, Dundee, for permission to have access to the patients under their care.

CONCLUSIONS.

The blood group of 50 cases of malignant disease has been determined, and the incidence of each compared with that in 125 cases of other diseases and 50 normal persons.

It is found that, while persons belonging to all four groups are liable to malignant disease, those in Groups I and III appear to be peculiarly susceptible and the clinical type of disease is generally speaking more malignant.

THE INFLUENCE OF BILE-SALTS UPON GASTRIC FUNCTION.

C. A. PANNETT AND C. M. WILSON.

From the Physiological Laboratory, St. Mary's Hospital, London.

Received for publication January 13th, 1921.

In certain operations performed upon man for some gastric disorders the bile is allowed free access to the stomach. An investigation was undertaken to determine whether any alterations in its motor or secretory functions take place as the result.

In the first place digestion in the stomach of the cat was inquired into. It was found that digestion, although inhibited in its onset, would take place when the animal was under the influence of urethane. It is true that such digestion is not quite normal, as the reflex excitation of gastric juice secretion through the higher senses is eliminated. It was, indeed, an investigation of the second chemical phase of digestion of Pawlow. But therein resides a certain merit in the method, for the disturbing effect of higher centre activity is removed, and with it one liability to error.

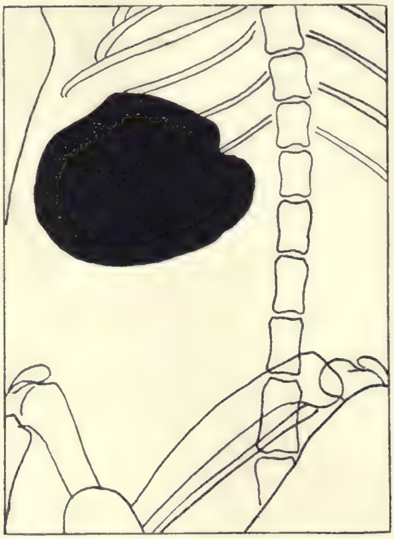


FIG. 1.

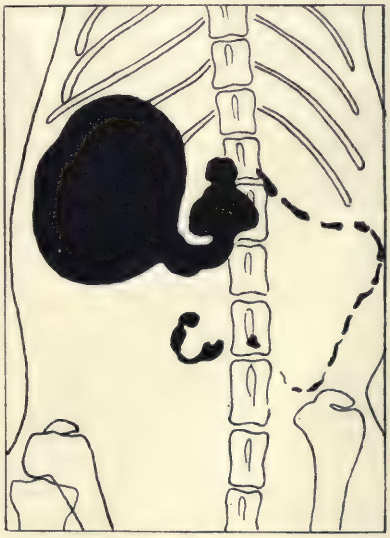


FIG. 2.

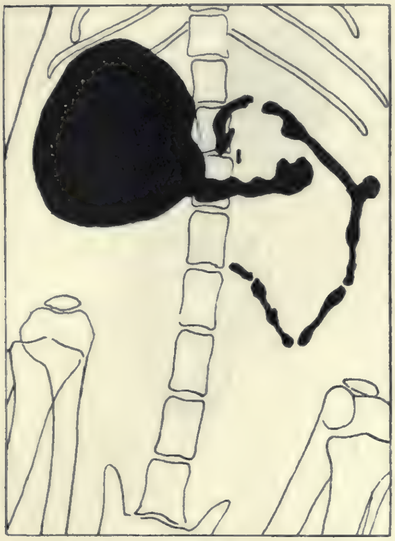


FIG. 3.

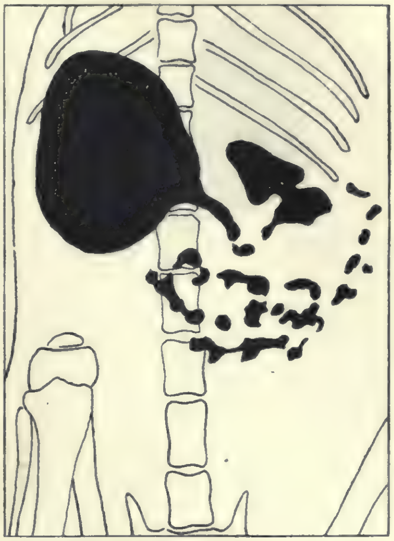


FIG. 4.

Experiment 1.—7/12/20: Tabby cat, weight, 3·5 kgrm. approximately; 4 grm. urethane subcutaneously. The following test-meal was administered by stomach-tube:

Rolled oats (1 oz. to 1 pint of water, boiled and filtered) . . .	50 c.c.
Barium sulphate . .	7·5 grm.

Skiagrams were taken 10 minutes, 25 minutes, 1 hour 20 minutes, 3 hours and 4 hours after the administration of the meal. Figs. 1 to 5 are tracings from the photographs. At the end of 4 hours there is still a considerable quantity of food in the stomach.

Experiment 2.—13/12/20: Same cat as in Experiment 1; 4 grm. urethrane subcutaneously. A similar meal given, with the addition of 0·175 grm. sodium taurocholate. Skiagrams were taken 7 minutes, 20 minutes, 35 minutes and 65 minutes after administration. Figs. 6 to 9 are the tracings. It will be seen that the stomach began to empty early, and the whole meal had passed the pylorus in a little over 1 hour.

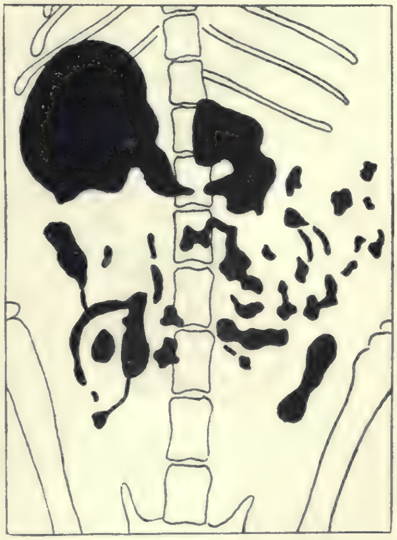


FIG. 5.

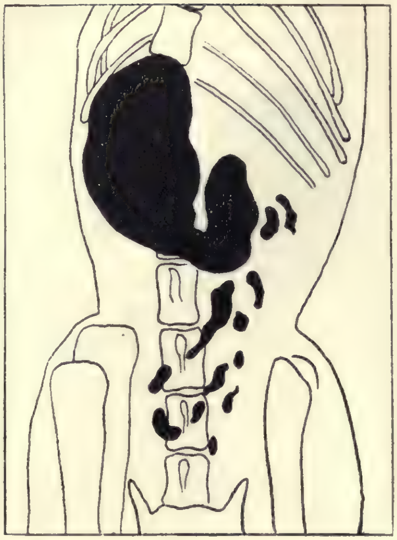


FIG. 6.

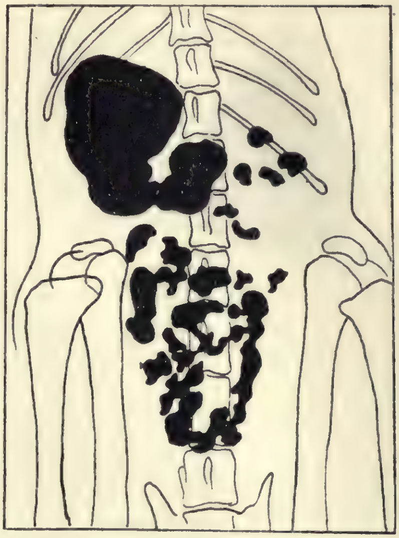


FIG. 7.

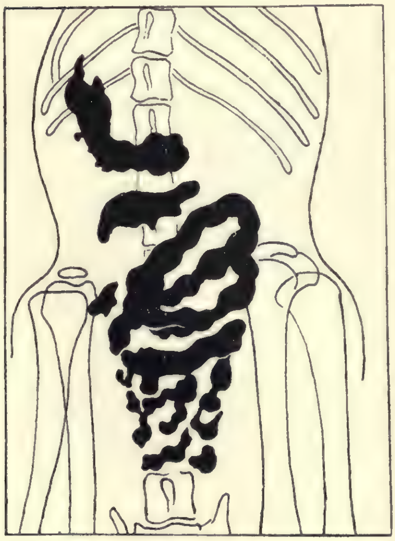


FIG. 8.

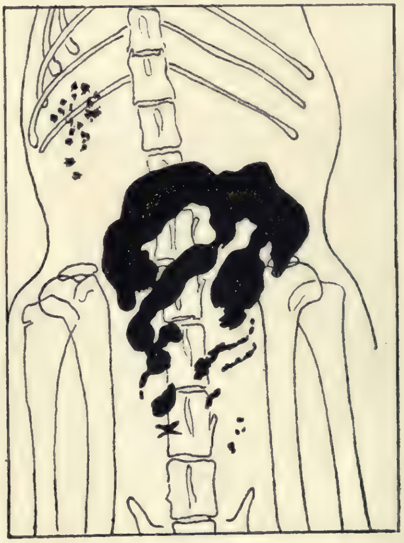


Fig. 9.

Experiment 3.—21/11/20: Black cat; 4 grm. urethane subcutaneously. Similar meal to that in Experiment 1. Screen observations.

Time, 12.30: Meal administered.
2 p.m.: None had passed the pylorus.
3 p.m.: About one-half of meal in the small intestine.
4 p.m.: Stomach almost empty.

Experiment 4.—28/11/20: Black cat; 4 grm. urethane subcutaneously. Similar meal to Experiment 2 administered. Skiagrams taken.

Time, 12 noon.: Meal administered.
12.10 p.m.: Food beginning to pass pylorus.
12.15 p.m.: Moderate quantity has passed into intestine.
12.30 p.m.: Considerable quantity in intestine.
1 p.m.: About one-half of meal has left stomach.

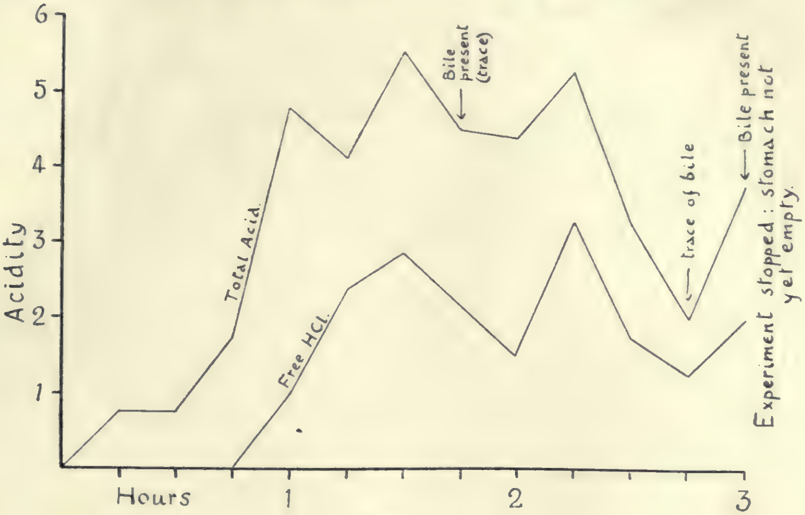


FIG. 10.

Stomach emptied some time between 1 p.m. and 1.55 p.m. No screen examination made between these times. Stomach quite empty at 1.55 p.m.

From these experiments it seems justifiable to conclude that the motor power of the stomach is greatly increased by the addition of sodium taurocholate to the gastric contents, and thereby a rapid evacuation takes place.

Corroborative evidence was obtained by employing the fractional test-meal method of investigation in a normal man.

Experiment 5.—20/11/20: C. A. P—, normal man. On an empty stomach a test-meal of 1 pint of gruel (rolled oats 1 oz. to water 1 pint) taken. Fractional estimation of stomach contents. Fig. 10 is the curve.

Experiment 6.—27/11/20: Same man as in Experiment 5. Same meal taken.

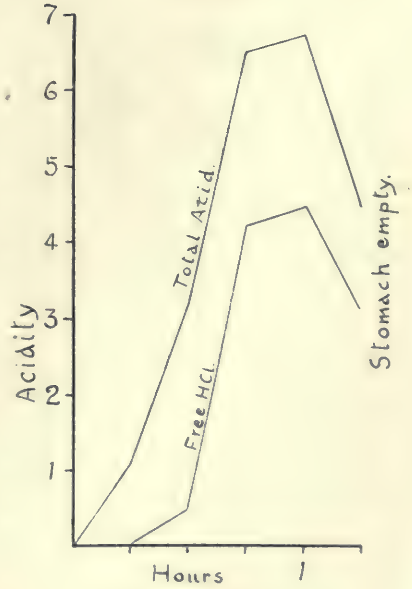


FIG. 11.

Then 1 grm. sodium taurocholate inserted into stomach down tube. The fractional curve is shown in Fig. 11.

From these experiments it will be seen that in a normal man also a slight addition of sodium taurocholate was followed by a rapid evacuation of the test-meal. The curve in Fig. 11 also shows a more rapid rise of acidity and an ultimate higher level. Whether or not this is a regular result and whether there is any alteration in digestive power of the juice secreted is at present the subject of further research.

CONCLUSIONS.

It is shown experimentally in cats and man that the addition of a small quantity of sodium taurocholate to a test-meal is followed by an abnormally rapid evacuation of the stomach contents.

THE ÆTIOLOGY OF RICKETS: AN EXPERIMENTAL INVESTIGATION.

D. NOËL PATON AND A. WATSON.

From the Institute of Physiology, University of Glasgow.

Received for publication December 2nd, 1920.

THE present investigation is a continuation of the experimental study of the ætiology of rickets which has been carried on in this laboratory since 1907, first by Leonard Findlay and later by Findlay, Noël Paton and Watson.

Since the publication of our last paper (1) some records of similar experimental work by Mellanby have appeared (2), and upon these a series of far-reaching conclusions have been formulated and published by the Vitamine Committee of the *Medical Research Council.*(3)

This report states "that rickets is a deficiency disease due to diets which are unbalanced in that they contain too little of those substances rich in an anti-rachitic factor and too much of those substances deficient in this respect. The anti-rachitic has, in many respects, a similar distribution to the fat-soluble A factor, and is possibly identical with this substance."

An examination of Mellanby's records shows that he reports none of the experiments upon which he bases the statement that "he cleared the way for his dietary experiments by a series of experiments to show that other factors were not operative." It is therefore impossible to estimate their value.

As to the influence of the dietary factors, he has recorded observations upon thirty-five pups, eleven normal and twenty-four which developed rickets. Presumably the experiments selected for publication were not the least convincing of his series. When the records are examined it is found that the dogs grouped together *were not always of the same litter.* Now, to contrast animals of different litters is, as we have stated before, wholly unjustifiable, since the susceptibility of different strains to rickets is notoriously different. Further, in his groups the pups were not always killed at the same age, and yet the calcium content of the bones is compared, although, as is known, this varies with age.

In this small series of observations no less than thirteen substances were tested and conclusions drawn as to which of these allows and which prevents the onset of rickets.

Our previously published work seems to show that such conclusions are unwarranted.

Findlay in 1908 (⁴) gave the result of his observations on the influence of diet and of confinement, from which he then came to the conclusion that the latter is the main factor in the development of rickets. That the condition developed was actually rickets was demonstrated by his histological examination of the bones, and the diagnosis was confirmed by Schmorl of Dresden, to whom the specimens were submitted.

Our observations published in 1918 were made upon seventeen puppies belonging to two litters. The main object was to get rickety pups and sound pups of the same litter and of the same age for chemical examination. But a test was also afforded of the relative influence of diet and open-air life.

From each litter two puppies were sent to the country, kept in a large yard and fed upon oatmeal porridge and skimmed milk, so that each had about 2·8 grm. of milk-fat per day—0·7 grm. per kilo. at the end of the experiment. Those kept in the laboratory had whole milk, containing, during the first period of their growth, about 3 grm. of butter-fat per day, and during the later period 6 grm.—about 2·4 grm. per kilo. at the end of the experiment.

In addition to this, three of the latter had butter, *not margarine*, in an amount to yield, along with the milk, 14 grm. of milk-fat *per diem.*

The puppies kept in the country on only 2·8 *grm. of milk-fat remained absolutely normal, whereas all the puppies kept in the laboratory developed rickets in spite of the ample supply of milk-fat* (*from* 3 *up to* 6 *grm.*), *and one of those receiving butter* (*in all* 14·5 *grm. of milk-fat*) *became most markedly affected.*

It has been suggested that puppies kept in the country get an anti-rachitic or fat-soluble substance by eating grass. But they did not eat grass, and further, the carnivorous alimentary canal does not digest grass even when swallowed. It has also been argued that confinement may so depress digestion and metabolism that the fat-soluble substance is not liberated or absorbed from the food, but there was no indication of disturbed digestion in the animals kept in the laboratory. Mellanby maintains that pups may be reared in confinement free from rickets if the diet contains an adequate supply of some anti-rachitic substance.

These observations seem to negative the view that rickets is due to the absence of a hypothetical anti-rachitic substance associated with milk-fat.

In this experiment the pups were given a very large amount of oatmeal and the energy intake was superabundant.

PRESENT INVESTIGATION.

Litters V and VI.

During the early months of 1920 observations were made upon two litters of pups with a more limited energy supply in the food.

The object of the experiment was to test the possibility of rearing dogs free from rickets in confinement on a diet rich in milk-fats with which the Vitamine Committee (³) maintain that an anti-rachitic accessory factor or vitamine is associated, and on one poor in these fats supposed to contain this hypothetical vitamine.

One observation on the effect of substituting dried milk for fresh milk was also made.

The diet consisted of a weighed quantity of oatmeal made into porridge, with, in some cases, a measured amount of whole milk and in others a similar amount of separated milk, and in one case an equivalent amount of whole cream dried milk (Cow and Gate brand).

The composition of the milks was as follows :

Whole milk.

	Grm.	Calories.
Fat	3·0	27
Protein	3·5	32·9
Lactose	4·5	
		59·9 per 100 grm.

Separated milk.

	Grm.	Calories.
Fat	0·33	2·1
Protein	3·5	32·9
Lactose	4·5	
		35 per 100 grm.

Dried whole cream milk (Cow and Gate), see p. 79.

The same weight of oatmeal and of milk, whole or separated, was given to each animal.

The first litter consisted of three pups which were put upon the diet at the age of ten weeks. One, A, had 30 grm. and later 45 grm. of oatmeal as porridge, with 250 c.c. of whole milk ; the second, B, had the same amount of oatmeal with 250 c.c. of skimmed milk ; while the third had the same quantity of oatmeal with milk made by dissolving 37 grm. of full cream dried milk (Cow and Gate brand).

The pups of this litter were killed at the age of twenty weeks. (Table I, Litter V, p. 86.)

The second litter consisted of six pups, which were put upon the diet at the age of five weeks. Two of these were put upon the same diet as A in the last series ; a third dog was put upon this diet for a month, when the milk was reduced to 150 c.c.

Another pup was put upon the same diet as B in the last series, and two others were put upon this diet for a month, when the milk was reduced to 150 c.c.

In both litters the oatmeal was increased from 30 grm. to 45 grm. at the end of a month.

Four of the pups of the second litter were killed at fifteen weeks old. (Table I, Litter VI, p. 86.)

In our experience, and apparently in that of Mellanby, rickets develops before the fifteenth week, and if the pup reaches that age without manifesting signs of the disease, it usually remains free from it, however it may be treated. We generally found that if rickets was going to develop it became manifest at about the twelfth week of life.

One pup, D 1, on 250 c.c. of whole milk, which at fourteen weeks of age showed slight signs of rickets by X-ray photography, and one on 150 c.c. of skimmed milk with well-marked rickets, were kept till nineteen weeks old. The former had the same allowance of whole milk, the latter had 250 c.c. of whole milk with 10 grm. of butter per day. When killed the rachitic condition was found in both cases to have advanced. The added butter-fat had produced no curative effect. (Plate II, figs. 7 and 8.)

Figs. 2, 4 and 6 (Plate I) show the condition of the bones as indicated by X-ray photographs in illustrative cases, and Figs. 1, 3 and 5 the appearance of some of the pups at the end of the experiment.

Photographs of the bones by X-ray and of all the pups at the end of the experiment are preserved in the laboratory and may be seen.

Table I (p. 86) gives the details of the experiments.

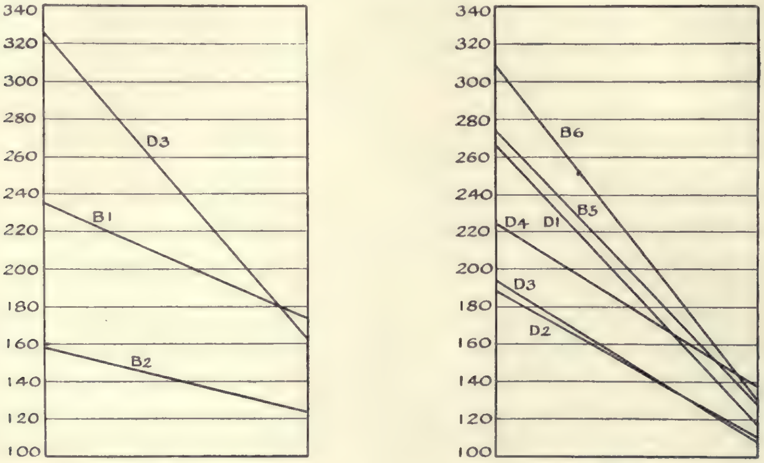


CHART 1. CHART 2.

Charts 1 and 2.—Showing energy value of diets in Calories per kilo. of body-weight throughout experiments on Litters V and VI. Litter V, D 3, very slight rickets. B 1, slight rickets. B 2, rickets. Litter VI, B 5 and D 1, slight rickets. B 6, D 4, D 3 and D 2, rickets.

Such a series of observations shows that:

(1) A diet containing 250 c.c. of whole milk—about 125 c.c. per kilo.—with no less than from 7·6 to 3 grm. of milk-fat per kilo. (see Table I) did not prevent the onset of rickets in pups kept in the laboratory, although they remained otherwise healthy, thus confirming our previous results.

(2) The pups on the separated milk diet and those on the reduced whole-milk diet of 150 c.c. developed rickets sooner and more markedly than those on the full whole-milk diet of 250 c.c. and on the dried milk diet.

Charts 1 and 2 show the energy value per kilo. throughout these experiments, the decrease in each case being due to the gain in weight of the animals. The graphs are simplified by the use of a straight line in place of a curve, the result of the rate of growth modified by the increase of oatmeal and, in some cases, by the reduction in the milk. The more complex curve can be readily constructed from the data given in the table.

It will be noticed that in both experiments the dogs with the lowest energy intake became the most markedly rachitic, with the exception of B 6, a small pup which grew little and which, although the energy intake was as great as that of B 5 and D 1, became distinctly rickety.

It is thus suggested that the development of rickets in pups fed upon oatmeal and separated milk, and the less rapid development upon oatmeal and whole milk is due not to the absence of any anti-rachitic factor but rather to a defective supply of energy in relationship to the rate of growth. A diet which contained sufficient energy for the slow-growing pup might be deficient for a more rapidly growing animal, and this might explain Mellanby's contention that rapidly growing animals are more susceptible to rickets.

In his experiments the effect of a diet of bread with skimmed milk is compared with that of one with bread and whole milk, the bread in both cases being given *ad libitum.* But it is difficult to get pups to eat more than a certain proportion of bread to milk, and hence the energy intake of the former diet must have been considerably less than the latter. While whole milk yields about 60 Calories per 100 grm., separated milk yields only about 35 Calories.

It is not suggested that a low energy value of the diet is the cause of rickets. The first and second experiments recorded by us show that pups on an ample diet may develop the disease. All that is suggested by this experiment is that a sufficient energy intake may decrease the susceptibility to the disease apart from the influence of any accessory factor, and this seemed to us to require further investigation.

Hess and Unger (5) have recorded observations on infants which suggest that the energy value of the food, in whatever proximate principle supplied, may have an important influence.

Litters VII, VIII and IX.

In order to test whether the onset of rickets may be retarded by giving an ample supply of energy apart from any accessory factor associated with milk-fat, the following experiment was arranged and carried out.

To secure that the diet should be uniform, dried milk (Cow and Gate brand) was used in place of sweet milk except in the case of three pups one of which died. The previous experiment had shown that dried milk may safely be substituted for sweet milk.

The samples of dried milk purchased were analysed and the energy value directly determined in the calorimeter.

	Fresh cow's milk.	Cow and Gate dried milk.	
		Full cream.	Separated.
Moisture	—	5·65	8·75
Ash	—	6·25	7·00
Fat	3·0 per cent.	21·00	2·08
Casein	3·5 ,,	27·35	34·36
Lactose	4·8 ,,	39·72	47·81

The bread was purchased in large quantities, dried, analysed and kept in a dry chamber.

Fortunately, three separate litters of pups, in all seventeen animals, were available at the same time, so that the effects of the treatment could be tested on different strains—a point of no little importance.

The plan of the experiment was to put groups of pups from the different litters, so arranged that some of each litter should be in each group, upon the different diets to be tested.

Litter.	No. in litter.	Born.	Put on diet.	Killed.	Age in weeks.
VII	8	15 : 3 : 20	19 : 4 : 20	13 : 7 : 20 (*D 1 and D 2 not killed*)	17
VIII	6	17 : 3 : 20	19 : 4 : 20	13 : 7 : 20 (*B 6 not killed*)	17
IX	3 (1 died, 23 : 6 : 20.)	21 : 3 : 20	21 : 4 : 20	5 : 7 : 20 (These died under anæsthetic)	15

In two sets of pups the diet contained an abundance of milk-fat, (*a*) in one in fresh milk, and (*b*) in the other in dried full cream milk (Cow and Gate). In the other sets the milk-fat was reduced to a mimimum by the substitution of separated dried milk (Cow and Gate) for whole milk, the energy thus withdrawn being made up by (*a*) in the one set, the addition of lard, a fat which the Vitamine Committee classify as devoid of their hypothetical anti-rachitic substance ([3], p. 83), and (*b*) in the other set, an increase in the amount of bread and of separated milk.

The diets were:

A. Whole fresh milk and bread.

B. Whole dried milk (Cow and Gate) and bread; the bread was the same in amount as in A, and the milk-powder in an amount yielding equal energy value with the whole milk.

C. Separated milk (Cow and Gate) of the same amount as in B, and with the deficient fat supplied by 10 grm. of lard, with the same amount of bread.

D. Separated milk (Cow and Gate) with bread.

The intention was to make up the energy value in group D to the same as the other groups by increasing the bread ration, but it was found impossible to get the pups to eat this, and the energy had to be supplied by increasing the separated milk to yield the energy required.

At the beginning of the experiment, when the pups were five weeks old, the diets were:

A.	Cow's milk (fresh)	170 c.c.
	Bread	30 grm.
B.	Full cream dried milk (Cow and Gate)	22·5 grm.
	Water	150 c.c.
	Bread	30 grm.
C.	Separated dried milk (Cow and Gate)	22·5 grm.
	Water	150 c.c.
	Bread	30 grm.
	Lard	4 grm.
D.	Separated dried milk (Cow and Gate)	22·5 grm.
	Water	150 c.c.
	Bread	40 grm.

This is equivalent to about 195 Calories.

As the pups grew the diets were increased so that the energy supplied per kilo. of pup remained as nearly as possible the same. The adjustment proved somewhat difficult, as is shown in the following table.

Energy Value of Diets per Kilo. at Different Ages.

Diet. Age in weeks	5.	8.	11.	14.	At end of experiment.
A. Sweet milk	190	248	256	237	237
B. Full cream	178	221	246	163	138
C. Separated and lard	186	218	242	212	169
D. Separated and bread	211	257	211	192	167
Average	191	236	239	202	177

At the age of eight weeks the diet was increased to the following amounts, on which the pups were kept till the age of eleven weeks:

A. Cow's milk (fresh)	255 c.c.
Bread	45 grm.
B. Full cream dried milk (Cow and Gate)	34 grm.
Water	225 c.c.
Bread	45 grm.
C. Separated dried milk (Cow and Gate)	34 grm.
Water	225 c.c.
Bread	45 grm.
Lard	6 grm.
D. Separated dried milk	34 grm.
Water	225 c.c.
Bread	60 grm.

This is equivalent to about 240 Calories per kilo.

At eleven weeks of age the diets were raised to those given below:

A. Cow's milk	340 c.c.
Bread	60 grm.
B. Full cream dried milk (Cow and Gate)	45 grm.
Water	225 c.c.
Bread	60 grm.
C. Separated dried milk (Cow and Gate)	45 grm.
Water	225 c.c.
Bread	60 grm.
Lard	8 grm.
D. Separated dried milk (Cow and Gate)	56 grm.
Water	375 c.c.
Bread	60 grm.

This is equivalent to about 213 Calories per kilo.

At fourteen weeks of age the pups were put upon the diets increased to the following amounts:

A. Cow's milk (fresh)	425 c.c.
Bread	75 grm.

B.	Full cream dried milk (Cow and Gate)	56 grm.
	Water	300 c.c.
	Bread	75 grm.
C.	Separated dried milk (Cow and Gate)	56 grm.
	Water	300 c.c.
	Bread	75 grm.
	Lard	10 grm.
D.	Separated dried milk	70 grm.
	Water	375 c.c.
	Bread	75 grm.

This is equivalent to about 205 Calories per kilo., and at the end to 180 Calories.

The energy intake was thus initially smaller, but later larger than in the previous Litters V and VI, and this is associated with more rapid growth.

In the separated milk diet the milk-fat per kilo. was initially smaller, and at the end not much greater than in Litters V and VI. *A substance associated with milk-fat did not appear to influence the rate of growth.*

Two pups on the separated milk and bread diet were kept in a large kennel with an open yard, and were freely exercised every day.

The risk of fæcal infection and the influence of lice or fleas were minimised by very special precautions as to cleanliness. One of the laboratory attendants devoted his whole time to the care of the animals, and the work was done most conscientiously. The pups in the laboratory were kept in a large, sunny room upon a concrete floor, enclosed in pens made of rabbit netting. Each pen measured 5 ft. 6 in. square. The pens were moved every day. The pups were fed separately—in groups—and weighed every week. In order to try to hasten the onset of rickets, two upon the diet of separated milk and bread were kept in a rabbit-hutch from twelve weeks of age to sixteen weeks of age. The man in charge found it extremely difficult to keep these two clean and free from vermin, but this was done by frequently changing the hutch, and the pups remained perfectly well.

Those on fresh milk, on account of their small average gain in weight, show a high energy intake to the end of the experiment. Those on dried full cream milk, on account of their marked gain in weight, show a distinct decrease of the energy intake, while those upon separated dried milk and lard, and those upon separated dried milk and bread, show a less decrease on account of their lesser increase in weight.

The experimental results are given in Tables II–VI (pp. 87 to 91), and the rate of growth and energy value of the diets in Charts 3–8 (pp. 91 to 94).

At the end of the experiments, when the pups of Lots VII and VIII were eighteen weeks of age and those of Lot IX fifteen weeks old, each had the two fore-legs X-rayed. They were then killed and the bones of one fore-leg were sent to Prof. Hopkins, while those of the other were used for examination of the epiphyseal cartilages.

Only one of these sixteen pups—VII, D 3—which had begun to lose weight before it was killed, developed rickets and the condition was not diagnosed till the X-ray photograph was examined. Figures of the condition of the right wrist in this and in another pup of the same litter and group (B 5) are given (Figs. 10 and 11, Plate II). The photographs of the others are not reproduced, but they are preserved in the laboratory and may be inspected.

These results do not seem capable of explanation on the theory that rickets is caused by the absence from the diet of an anti-rachitic factor associated with milk-fat.

It might be urged that the separated milk may contain an amount of an anti-rachitic factor sufficient to prevent the onset of the disease. But the basis of Mellanby's experimental observations is the finding that separated milk up to 250 to 350 c.c. and white bread even with linseed oil, yeast and orange juice will not prevent the onset of rickets in pups between six weeks and twelve weeks of age, while whole milk will prevent it (2), and upon this is based the practical recommendation of the Vitamine Committee that full-cream milk should be used in children for the prevention of the disease (3, p. 99).

Butter is placed with cod-liver oil (3, p. 102) as the substance richest in "fat-soluble A or anti-rachitic factor," and yet in our Experiments I and II butter failed to prevent the onset of rickets and it failed to cure it in a pup of Litter VI. Spontaneous recovery from rickets occurs in dogs as in children, and the recorded cases of "cure" by the administration of butter fat may have been due to this.

Our previous experience has taught us how difficult it is to rear pups free of rickets in a laboratory, in spite of the most generous diets. The results of a large series of observations recorded by Renton and M. Robertson (6) is a striking illustration of this.

The outbreak of rickets in a kennel of fox-hounds at Adelaide, recorded by Bull (7), in which removal of some of the pups to fresh ground prevented the onset of the disease, suggests very strongly that some bacterial infection plays a part in the development of rickets, possibly of the same non-specific character as that which the investigations of McCarrison have shown to be the causal factor in goitre. Morpurgo's (8) results upon young white rats also point in the same direction.

The experiment recorded in the *British Medical Journal* in 1908 (4) shows that pups kept in the country on a fat-poor diet may escape rickets, while those of the same litter, segregated in a laboratory on a fat-rich diet, may develop it.

The first experiment of the present series definitely confirms the experience that under usual laboratory conditions an abundant supply of milk-fat may fail to prevent the onset of rickets and that the addition of butter-fat to the diet has no curative action. It shows that dried milk may be substituted for fresh milk with safety in the diet of pups.

It suggests that a low energy intake may predispose to the development of the disease.

The last experiment shows that by scrupulous care as to cleanliness pups may sometimes be reared in a laboratory without developing rickets, on a diet of separated dried milk and bread, if an ample supply of energy—not necessarily in fats supposed to contain an anti-rachitic factor—is afforded.

The records of growth show that the rate is not strictly proportionate to the energy value of the diet, but that it is modified by other factors (Table VI).

The breed of the dog seems to play a part. The size of the fathers of the different litters is not known, but the pups of Litter VII, from a large, heavy collie, gained on an average 235 grm. per week, those of Litter VIII, from a smaller sheep collie, 170 grm. per week, and those of Litter IX, from a large, old collie, 146 grm. per week.

Our records also indicate that *pups kept in the open air grow more rapidly than those confined in the laboratory.* Chart 9 (p. 94), which gives the rate of growth of the pups used in Experiment I (1) and a comparison of Chart 8 with Charts 6 and 7 show this very clearly.

CONCLUSIONS.

(1) In young dogs under ordinary laboratory conditions a liberal allowance of milk-fat, up to even 14 grm. per kilo. of body-weight, neither prevents the onset of rickets nor cures it when it has developed.

(2) Pups kept largely in the open air may escape the development of rickets on an intake of less than 1 grm. of milk-fat per kilo. of body-weight.

(3) With scrupulous care as to cleanliness it is, at least sometimes, possible to rear pups free of rickets in the laboratory on an intake of only about 0·5 grm. of milk-fat per kilo. of body-weight, along with bread, provided that the diet affords an adequate supply of energy.

(4) The energy value of the diet, however supplied, quite apart from the presence of any hypothetical anti-rachitic factor in milk-fat, would seem to play a part in controlling the development of rickets, but that it is only a contributory part is shown by the development of rickets in pups with a high energy intake if confined in the laboratory without scrupulous care as to cleanliness.

(5) Milk-fat may be reduced to about 0·3 grm. per kilo. of body-weight, if its place is taken by an equal amount of lard, without the onset of rickets.

(6) The results of these obervations do not support the conclusion of the Accessory Food Factors Committee of the Medical Research Council that rickets is a deficiency disease due to a lack of an anti-rachitic factor associated with milk-fat.

REFERENCES.

1. Noël Paton, D., Findlay, L., and Watson, A.—"Observations on the Cause of Rickets," *Brit. Med. J.*, 1918, **2**, 625.
2. Mellanby, E.—"An Experimental Investigation on Rickets," *Lancet*, 1919, **1**, 407.
3. "Report on the Present State of Knowledge concerning Accessory Food Factors (Vitamines)," compiled by a Committee appointed jointly by the Lister Institute and Medical Research Committee. *Med. Res. Com., Spec. Rep. Series*, No. 38, London, 1919 (H.M. Stationery Office).
4. Findlay, L.—"The Ætiology of Rickets: a Clinical and Experimental Study," *Brit. Med. J.*, 1908, **2**, 13.
5. Hess, A. F., and Unger, L. J.—"The Clinical *Rôle* of the Fat-soluble Vitamin: Its Relation to Rickets," *J. Amer. Med. Assoc.*, 1920, **74**, 217.
6. Renton, J. M., and Robertson, Madge E.—"Thymusectomy and its Relationship to Rickets," *J. Pathol. and Bacteriol.*, 1916–17, **21**, 1.

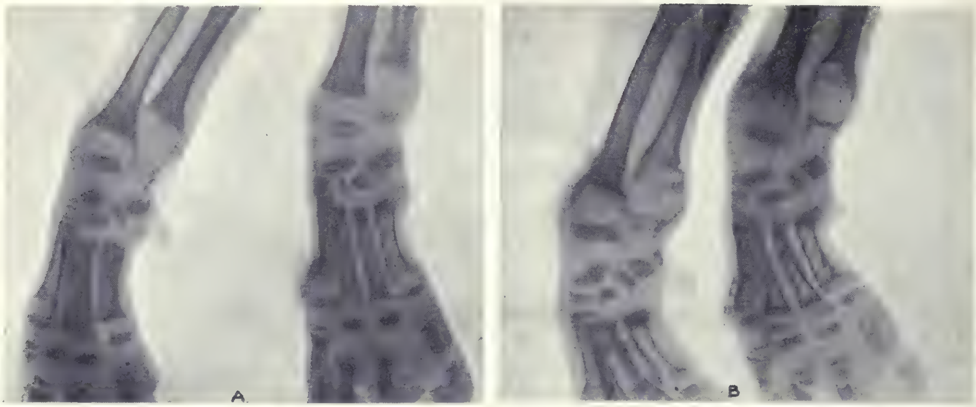


FIG. 8.

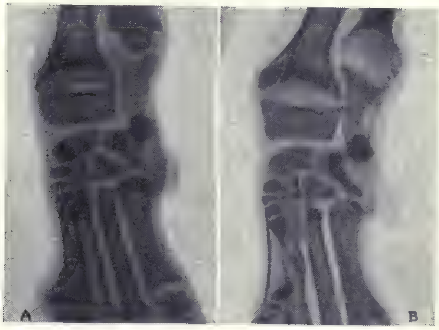


FIG. 7.

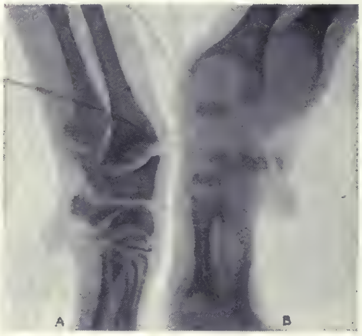


FIG. 9.

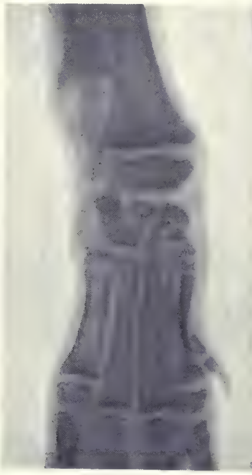


FIG. 10.

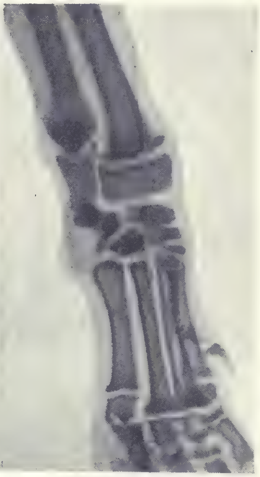


FIG. 11.

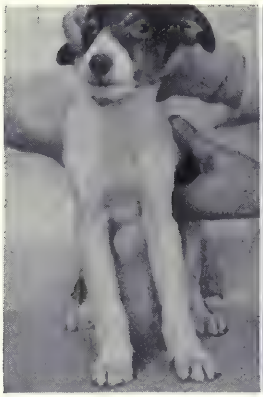


FIG. 1.

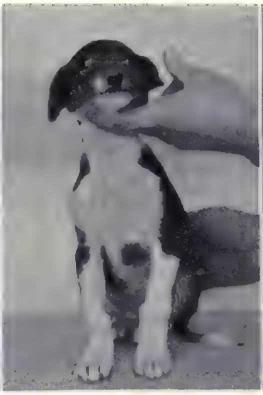


FIG. 3.

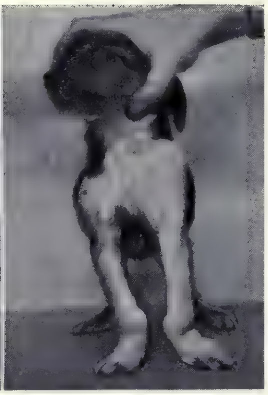


FIG. 5.

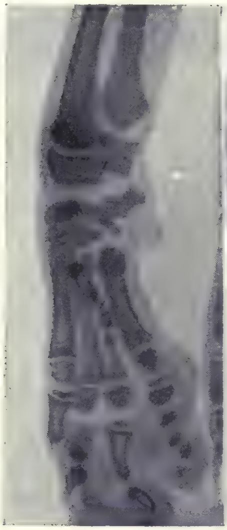


FIG. 2.

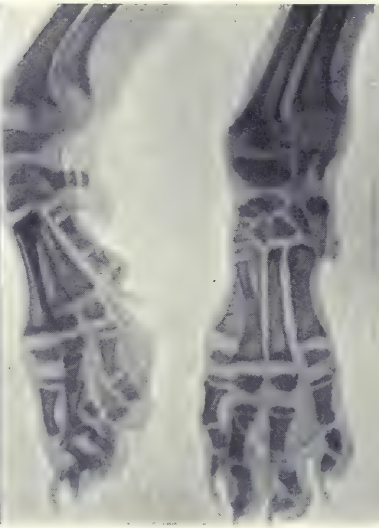


FIG. 4.

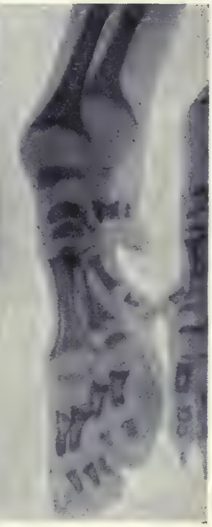


FIG. 6.

7. Bull, L. B.—"Rickets in Foxhound Puppies: a Clinical and Experimental Investigation," *J. Compar. Pathol. and Therap.*, 1918, **31**, 193.
8. Morpurgo, B.—"Durch Infection herforgerufene malacische und rachitische Skeletveränderungen an jungen Weissen Ratten," *Centralbl. f. allg. Pathol. u. pathol. Anat.*, 1902, **33**, 113.

The expenses of this investigation were defrayed by the Medical Research Council, to which our thanks are due.

EXPLANATION OF PLATES.

PLATE I.

Figs. 1 and 2.—Litter V, Dog 3, fed on oatmeal porridge and dried full-cream milk = 11 grm. milk fat. Energy value of diet 377 C. at start, 437 C. at end.

Figs. 3 and 4.—Litter V, Bitch 1, fed on oatmeal porridge and fresh whole milk = 7·5 grm. milk fat. Energy value of diet 270 C. at start, 330 C. at end.

Figs. 5 and 6.—Litter V, Bitch 2, fed on oatmeal porridge and separated milk, 0·8 grm. milk fat. Energy value 207 C. at start, 247 C. at end.

PLATE II.

Fig. 7.—Litter VI, Dog 1: a. Skiagram on 11:2:20 after being kept on porridge and 250 c.c. whole milk. b. Skiagram on 10:3:20 after another month on this diet. Note marked advance in the rachitic condition.

Fig. 8.—Litter VI, Dog 4.: a. Skiagram on 11:2:20 after being kept upon porridge and 150 c.c. separated milk. b. Skiagram on 10:3:20 after being kept for a month upon porridge with 250 c.c. whole milk and 10 grm. butter = 15·5 grm. milk-fat. Note advance in rachitic condition.

Fig. 9.—Litter VI: a. Bitch 5 and b. Dog 2, both on oatmeal porridge, but the former with 250 c.c. whole milk = 7·5 grm. milk-fat, energy value 270 C. at start, 330 C. at end; the latter with 250 c.c. separated milk = 0·8 grm. milk-fat, energy value 207 C. at start, 217 C. at end. Note much more marked rickets in the second.

Fig. 10.—Litter VII, Dog 3: Kept under the same conditions as Bitch 5. The only pup of nine from three litters which developed rickets on this diet.

Fig. 11.—Litter VIII, Bitch 5: Skiagram after being kept on bread and dried separated milk = 0·48 grm. milk-fat, energy value 364 C. at start, 549 C. at end. Note absence of rickets in this as in 8 other pups on the same diet.

TABLE I.

No.	Diet.	Milk-fat per day. Total.	Per kilo. First.	Per kilo. Last.	Energy value in calories. For first 4 weeks.	Energy value in calories. For last 6 weeks.	Weight in grm. Initial.	Weight in grm. Terminal.	Increase in weight. Per cent. of initial.	Increase in weight. Average per week from 10 to 20 weeks.	Condition when killed.
	LITTER V.										
	(Each had 30 grm. oatmeal for 5 weeks, afterwards 45 grm.)										
B 1	+ 250 c.c. whole milk (fresh)	7·5	6·5	3·9	270	330	1150	1900	65·2	75	Rickets (condition between B 2 and D 3).
B 2	+ 250 c.c. separated milk	0·8	0·6	0·4	207	247	1300	2000	53·8	70	Marked rickets.
D 3	+ 30 grm. dried whole milk	11·0	9·0	4·1	377	437	1150	2700	134·7	155	Slight rickets.
	LITTER VI.										
	Whole milk.									(From 5 to 15 weeks.)	
B 5	+ 250 c.c. whole milk	7·5	7·6	2·9	270	330	990	2575	160·1	158·5	Slight rickets.
D 1	+ „ „ „	7·5	7·3	2·7	270	330	1020	2770	171·5	175	Slight rickets. (Kept alive.)
B 6	+ „ „ „ then 150 c.c.	7·5 then 4·5	8·5	2·2	270	270	880	2070	135·2	119	Rickets.
	Separated milk.										
D 2	+ 250 c.c. separated milk	0·8	0·72	0·32	207	267	1100	2450	140·7	135	Rickets.
D 3	+ 250 c.c. separated milk, then 150 c.c.	0·8 then 0·48	0·74	0·22	207	232	1080	2121	96·3	104	Rickets.
D 4	+ 250 c.c. separated milk, then 150 c.c.	0·8 then 0·48	0·87	0·22	207	232	920	1680	82·6	76	Rickets. (Kept alive. Sweet milk and butter.)

TABLE II.—*Litters VII, VIII and IX (5th to 8th week of age).*

No.	Diet.	Milk-fat per day.			Energy value in calories.			Body-weight in grm.		
		Total.	Per kilo. Initial.	Per kilo. Terminal.	Total.	Per kilo. Initial.	Per kilo. Terminal.	Initial.	Terminal.	Gain per cent.
Lot VII, B 8	A.—Whole milk 170 c.c.; bread 30 grm.	4·6	3·83	3·17	215·26	179·4	148·5	1200	1450	20·8
Lot VIII, B 6			4·28	3·91		200·2	183·2	1075	1175	9·3
Lot VII, D 2	B.—Full cream milk (dried) 22·5 grm.; bread 30 grm.	4·5	3·0	2·1	221·71	138·5	105·5	1600	2100	31·0
,, D 5			3·5	2·7		173·8	134·3	1275	1650	29·0
Lot IX, D 1			4·5	3·8		221·7	188·6	1000	1175	17·5
Lot VII, D 1	C.—Separated milk (dried) 22·5 grm.; bread 30 grm.; lard 4 grm.	0·45	0·26	0·2	237·0	139·4	110·2	1700	2150	26·0
,, D 4			0·33	0·27		178·8	143·6	1325	1650	24·0
Lot VIII, D 2			0·4	0·31		204·7	163·4	1125	1450	28·9
,, B 4			0·4	0·4		215·4	215·4	1100	1100	0·0
Lot VII, D 3	D.—Separated milk (dried) 22·5 grm.; bread 40 grm.	0·45	0·33	0·28	243·0	180·0	154·2	1350	1575	16·0
,, B 7			0·4	0·32		221·0	174·8	1100	1390	26·0
Lot VIII, D 3			0·4	0·32		216·0	174·8	1125	1390	23·0
,, B 5			0·4	0·34		216·0	183·8	1125	1300	15·0
Lot IX, D 2			0·36	0·28		190·6	154·3	1275	1575	23·5
,, D 3			0·39	0·31		211·3	160·6	1150	1450	26·0
,, D 4			0·33	0·26		180·0	143·0	1350	1700	25·9
,, B 5			0·4	0·32		226·0	176·7	1075	1375	27·0
,, B 6			0·38	0·33		207·0	180·0	1175	1350	14·8

TABLE III (*8th to 11th week of age*).

No.	Diet.	Milk-fat per day.			Energy value in calories.			Body-weight in grm.		
			Per kilo.			Per kilo.				
		Total.	Initial.	Terminal.	Total.	Initial.	Terminal.	Initial.	Terminal.	Gain per cent.
Lot VII, B 8	A.—Whole milk 225 c.c.; bread 45 grm.	7·2	4·96	4·11	322·89	223·0	184·0	1450	1750	20·6
Lot VIII, B 6			6·11	4·7		274·0	211·0	1180	1530	30·0
Lot VII, D 2	B.—Full cream milk (dried) 34 grm.; bread 45 grm.	6·75	3·21	2·33	343·0	163·3	118·0	2100	2900	38·0
" D 5			4·09	3·0		208·0	152·0	1650	2250	36·3
Lot IX, D 1			5·77	5·2		292·0	264·0	1175	1300	11·1
Lot VII, D 1	C.—Separated milk (dried) 34 grm.; bread 45 grm.; lard 6 grm.	0·675	0·31	0·22	355·45	165·0	118·0	2150	3000	39·3
" D 4			0·41	0·26		215·0	139·0	1650	2550	54·5
Lot VIII, D 2			0·47	0·35		245·0	182·0	1450	1950	34·5
" B 4			0·61	0·5		323·0	263·0	1100	1350	22·7
Lot VII, D 3	D.—Separated milk (dried) 34 grm.; bread 60 grm.	0·675	0·43	0·28	364·45	231·0	152·0	1580	2400	51·9
" B 7			0·48	0·26		260·0	143·0	1400	2550	82·1
Lot VIII, D 3			0·48	0·29		260·0	158·0	1400	2300	64·3
" B 5			0·52	0·39		280·0	208·0	1300	1750	34·6
Lot IX, D 2			0·43	0·3		231·4	167·2	1575	2180	38·4
" D 3			0·47	0·35		251·0	192·0	1450	1900	31·0
" D 4			0·39	0·3		214·3	165·6	1700	2200	29·4
" B 5			0·49	0·39		264·0	208·0	—	1750	—
" B 6			0·5	0·36		269·9	194·0	1350	1875	38·8

Table IV (*11th to 14th week of age*).

No.	Diet.	Milk-fat per day.			Energy value in calories.			Body-weight in grm.		
			Per kilo.			Per kilo.				
		Total.	Initial.	Terminal.	Total.	Initial.	Terminal.	Initial.	Terminal.	Gain per cent.
Lot VII, B 8	A.—Whole milk 340 c.c.; bread 60 grm.	9·2	5·25	4·2	430·5	246·0	195·7	1750	2200	25·7
Lot VIII, B 6			6·03	3·9		282·3	183·2	1525	2350	54·0
Lot VII, D 2	B.—Full cream milk (dried) 45 grm.; bread 60 grm.	9·0	3·1	2·1	443·4	152·9	106·8	2900	4150	42·0
,, D 5			4·0	3·0		197·0	147·8	2250	3000	30·0
Lot IX, D 1			6·9	6·0		141·0	295·6	1300	1500	15·3
Lot VII, D 1	C.—Separated milk (dried) 45 grm.; bread 60 grm.; lard 8 grm.	0·9	0·45	0·25	473·9	130·0	131·6	2000	3600	20·0
,, D 4			0·35	0·23		185·8	118·5	2550	4000	56·8
Lot VIII, D 2			0·46	0·34		243·0	186·1	1950	2600	33·8
,, B 4			0·67	0·47		351·0	249·4	1350	1900	41·0
Lot VII, D 3	D.—Separated milk (dried) 56 grm.; bread 60 grm.	1·12	0·47	0·34	443·5	148·8	134·4	2400	3300	37·5
,, B 7			0·44	0·30		173·9	119·8	2550	3700	45·0
Lot VIII, D 3			0·49	0·37		193·6	147·8	2290	3000	31·0
,, B 5			0·64	0·41		253·4	164·2	1750	2700	54·2
Lot IX, D 2			—	—		—	—	2180	—	—
,, D 3			0·59	0·51		233·4	197·1	1900	2250	18·4
,, D 4			0·51	0·39		201·5	155·6	2200	2850	29·5
,, B 5			0·73	0·47		253·4	201·5	1750	2200	25·7
,, B 6			0·60	0·43		236·5	169·2	1875	2620	39·7

TABLE V (*after* 14 *weeks of age*).

No.	Diet.	Milk-fat per day.			Energy value in calories.			Body-weight in grm.		
		Total.	Per kilo. Initial.	Per kilo. Terminal.	Total.	Per kilo. Initial.	Per kilo. Terminal.	Initial.	Terminal.	Gain per cent.
Lot VII, B 8	A.—Whole milk 425 c.c.; bread 75 grm.	12·75	5·80	5·10	562·6	256	225	2200	2500	13·6
Lot VIII, B 6			5·42	4·55		239	201	2350	2800	19·1
Lot VII, D 2	B.—Full cream milk (dried) 56 grm.; bread 75 grm.	11·25	2·71	2·37	571·7	138	120	4150	4750	14·5
,, D 5			3·75	3·21		190·5	163	3000	3500	16·0
Lot IX. D 1			—	—		—	—	—	—	—
Lot VII, D 1	C.—Separated milk (dried) 56 grm.; bread 75 grm.; lard 10 grm.	1·12	0·31	0·27	592·4	165·5	143	3600	4150	15·3
,, D 4			0·28	0·24		148	128	4000	4625	15·6
Lot VIII, D 2			0·43	0·37		228	197	2600	3000	15·4
,, B 4			0·59	0·47		312	249	1900	2375	25·0
Lot VII, D 3	D.—Separated milk (dried) 70 grm.; bread 75 grm.	1·4	0·42	0·37	549·1	167	144·5	3300	3800	15·0
,, B 7			0·38	0·31		148	123	3700	4460	20·5
Lot VIII, D 3			0·47	0·37		183	144·5	3000	3800	26·6
,, B 5			0·52	0·41		203	163	2700	3375	25·0
Lot IX, D 2*			—	—		—	—	—	—	—
,, D 3			0·62	0·74		244	289	2250	1900	−15·5
,, D 4			0·49	0·43		192·6	170·2	2850	3225	13·0
,, B 5			0·64	0·56		250	220	2200	2500	13·6
,, B 6			0·53	0·48		209·6	190·6	2620	2880	10·0

* Died.

TABLE VI.—*Average gain per week on diets—*

A.	Lot VII,	B 8	118·0 grm.,	11 weeks.
	Lot VIII,	B 6	144·0 ,,	12 ,,
B.	Lot VII,	D 2	286·0 ,,	11 ,,
	Lot VII,	D 5	202·0 ,,	11 ,,
	Lot IX,	D 1	55·5 ,,	died at end of 9th week of diet.
C.	Lot VII,	D 1	222·7 ,,	11 weeks.
	,,	D 4	300·0 ,,	11 ,,
	Lot VIII,	D 2	156·0 ,,	12 ,,
	,,	B 4	106·0 ,,	12 ,,
D.	Lot VII,	D 3	222·7 ,,	11 ,,
	,,	B 7	305·0 ,,	11 ,,
	Lot VIII,	D 3	222·9 ,,	12 ,,
	,,	B 5	187·5 ,,	12 ,,
	Lot IX,	D 2	142·8 ,,	11 ,,
	,,	D 3	68·0 ,,	11 weeks; going down in weight before death.
	,,	D 4	158·0 ,,	11 weeks.
	,,	B 5	129·5 ,,	11 ,,
	,,	B 6	155·0 ,,	11 ,,

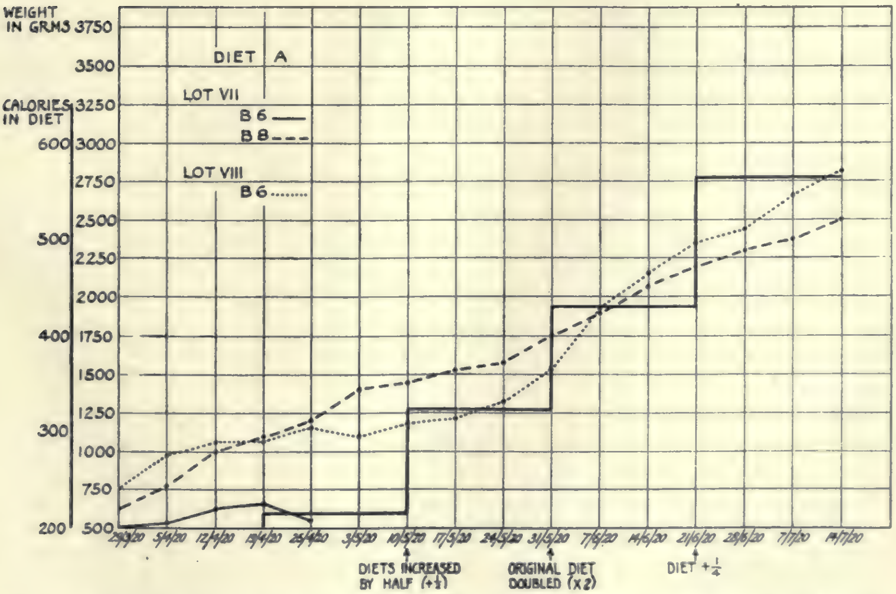


CHART 3.—Growth curves and energy value of diet of pups on fresh whole milk and bread.

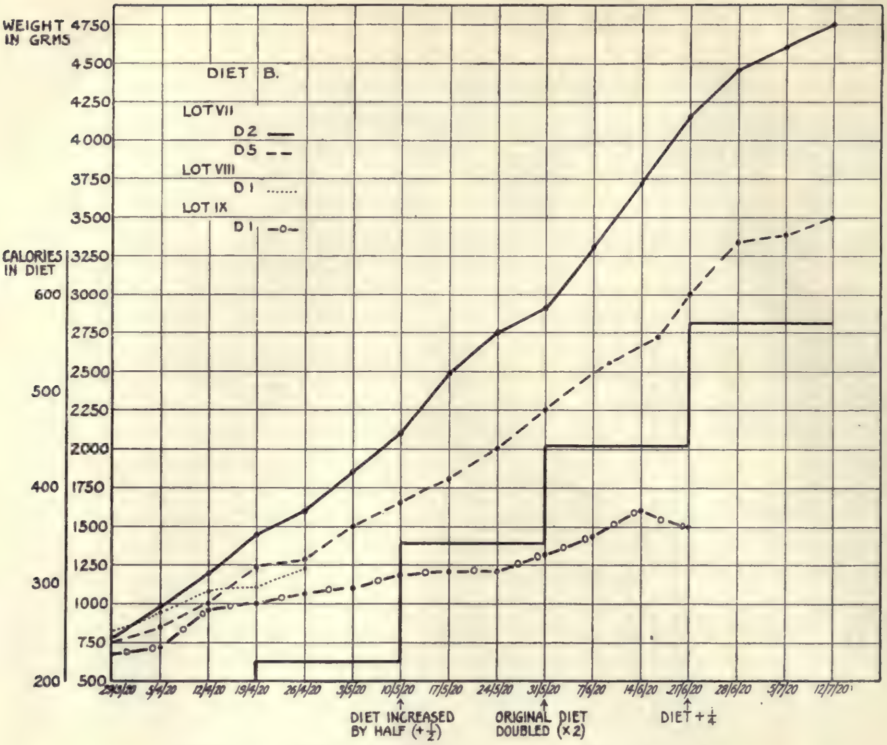


Chart 4.—Ditto on whole cream, dried milk and bread.

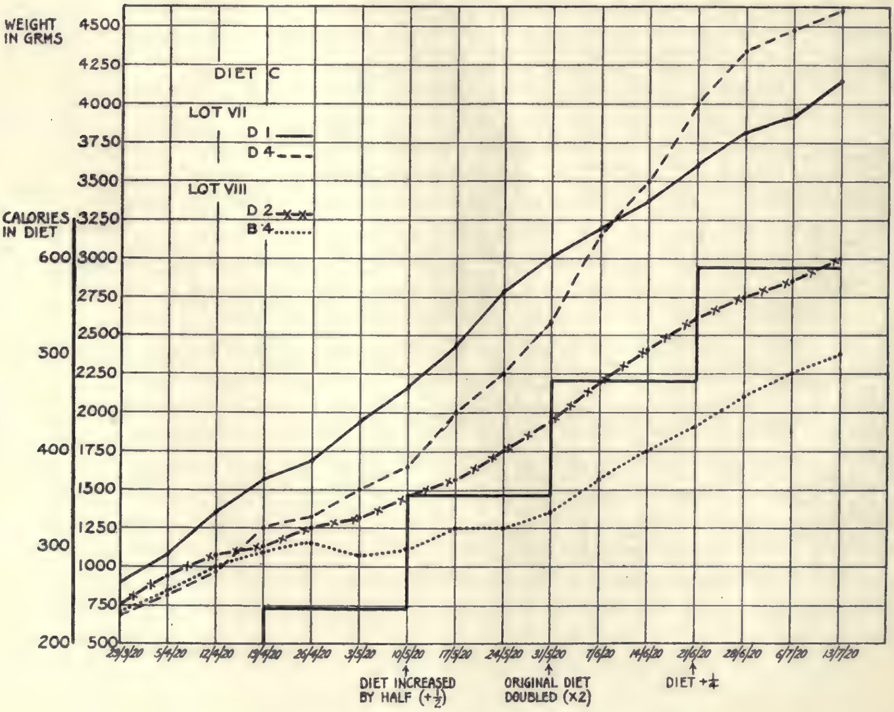


Chart 5.—Ditto on separated dried milk and bread with lard.

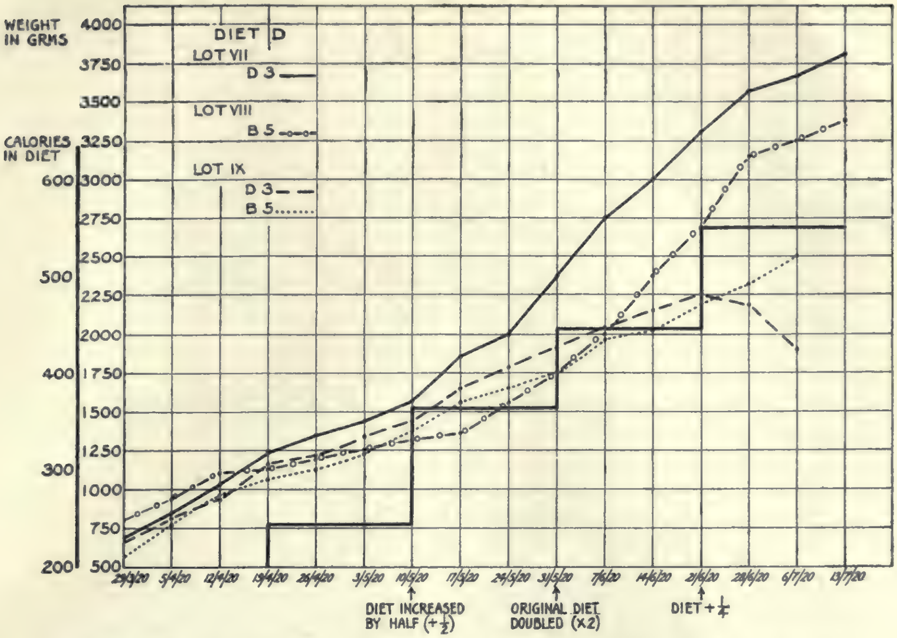


Chart 6.—Ditto on separated milk and bread, the pups being kept in a large pen in the laboratory.

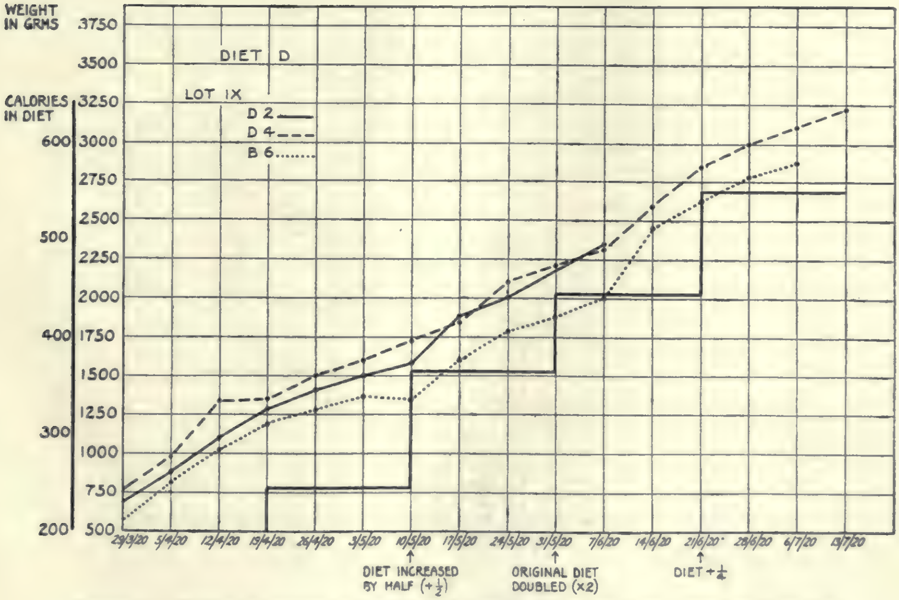


Chart 7.—Ditto on separated milk and bread, the pups being confined for the last three weeks of life.

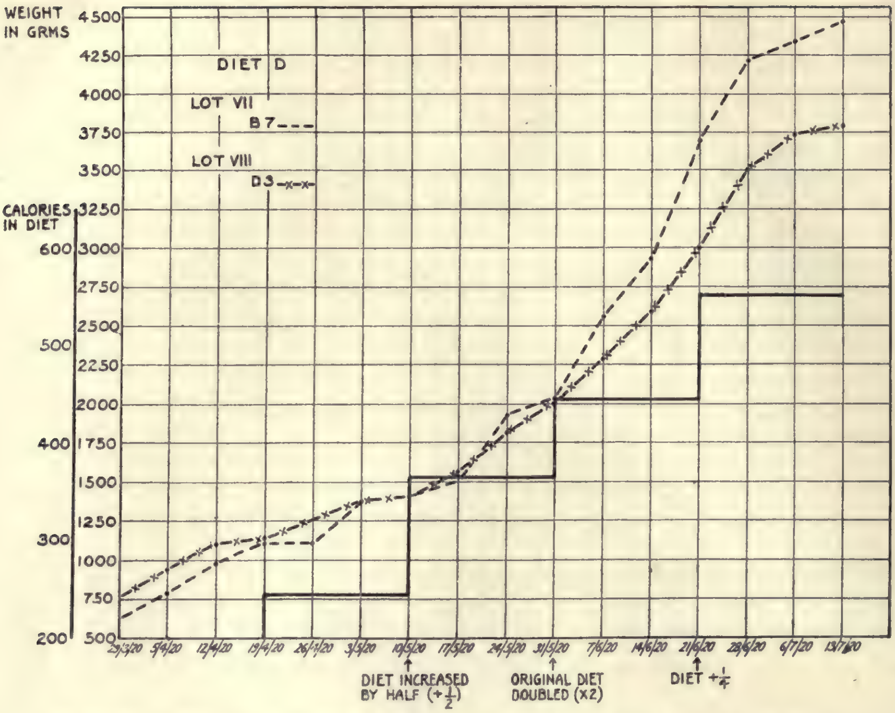


Chart 8.—Ditto on separated dried milk and bread, the pups being kept in the open air. Compare with D 3 and B 5 in Chart 6.

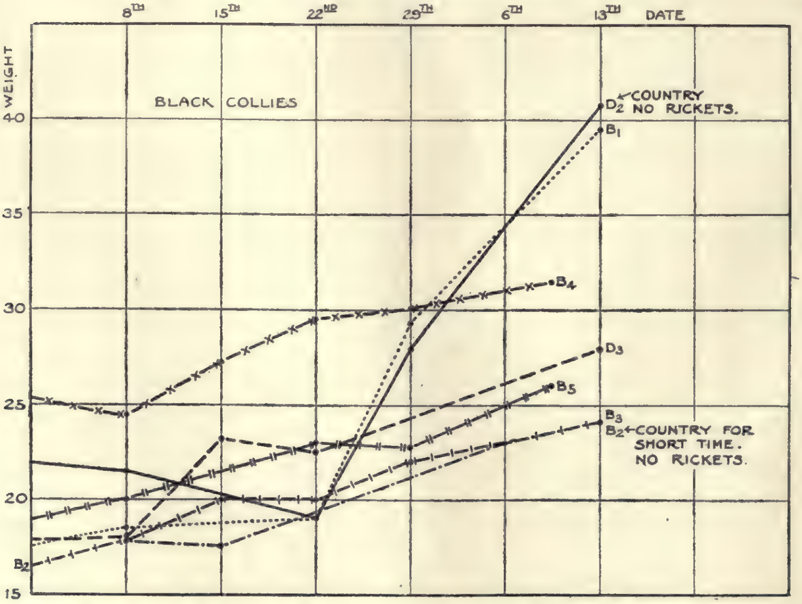


Chart 9.—Growth curves of pups of Experiment I recorded in *British Medical Journal* (1). D 2 and B 1 kept in the open air. B 2 kept in the open air for two weeks and then returned to the laboratory. None of these developed rickets. The others kept in the laboratory all developed rickets.

THE ANTISEPTIC POTENCY OF ACRIFLAVINE, WITH CONSIDERATIONS ON THE VARIABILITY OF RESULTS IN TESTING ANTISEPTICS.

C. H. BROWNING AND R. GULBRANSEN.

From the Pathological Department of the University and Western Infirmary, Glasgow, and the Bland-Sutton Institute of Pathology, Middlesex Hospital, London.

[A report to the Medical Research Council.]

Received for publication February 10th, 1921.

THE method very commonly adopted in defining the value of an antiseptic is to express its potency as the ratio of the reciprocals of the sterilising concentrations of the substance in question and some standard compound, commonly phenol. This procedure probably owes its acceptance in considerable part to the fact that it appears to eliminate irregularities due to variations, which are observed when the sterilising concentration of any single substance is determined on repeated occasions. In the case of the ratio, it is assumed that alterations in the resistance of the test organism, whether due to differences in the nutrient medium or to other causes, will affect the results with different antiseptics comparably and in the same direction, so that repeated experiments with the same pair of substances will yield a constant value. A method of this kind does not take into account the fact that alterations in hydrogen-ion concentration of the solution may affect the potency of different antiseptics in opposite directions, *e. g.* the action of mercuric chloride is enhanced when the reaction passes to the acid side, whereas the flavine compounds act best in more alkaline medium, as we have shown (3). A further circumstance which may interfere with the utility of the ratio as an expression of the value of an antiseptic, is that it is commonly calculated from experiments in which the period of contact of the organisms with the antiseptic agent is limited to a few minutes. Certain substances, *e. g.* phenol and mercuric chloride, act rapidly. On the other hand, there are many powerfully antiseptic organic compounds, such as those of the diamino-acridene group (" flavines "), which cause death of the organisms comparatively slowly; but, short of lethal action, they exert a powerful inhibitory or " bacteriostatic " effect, which can be advantageously utilised in therapy, *e. g.* in the case of infected wounds (1, 2).

When the preparation of the flavine compounds was first commenced on a commercial scale, it appeared to us advisable to make a careful comparison of the biological properties of different specimens, since organic chemical syntheses under conditions such as are involved in the production of these substances might give rise to mixtures including the products of side-reactions, whose behaviour on the tissues and on bacteria had not been investigated. The results showed that, as regards antiseptic power, a series of about forty commercial products did not differ from each other notably even when some impurity, in the form of insoluble matter, was present in varying degree. On the other hand, marked differences were found in irritating properties, as tested on the conjunctiva; this is probably a point of some considerable importance when the antiseptic is to be brought into contact with a very sensitive mucous membrane, such as that of the urethra. These systematic tests led to the accumulation of an extensive series of data regarding the absolute concentrations of diaminoacridene compounds which serve to inhibit growth and to sterilise inoculated media. The same compounds have been tested repeatedly and also different specimens have been compared. The results are now detailed in the case of those specimens of diaminoacridene methochloride—"acriflavine"—which have been most frequently examined, as they illustrate the range of variation which may be expected in a series of tests of this kind.

The tests were carried out in serum and in dilute peptone water. Serum was employed as it represents the fluid constituent to which antiseptics in contact with the tissues are exposed; also, it is to be regarded as extremely constant in composition and reaction, which are important properties in a medium employed in testing antiseptic action. The peptone water solution consisted of 0·7 per cent. bacteriological peptone along with 0·35 per cent. sodium chloride. At first the important effect of small alterations in the hydrogen-ion concentration of this solution was not appreciated and the earlier tests were made on a medium with a P_H value below 6; subsequently a solution with a P_H value of 7·2 to 7·8, approximately, was employed. The striking increase of antiseptic potency in the more alkaline medium is well shown in Tables III and IV. The experiments with *B. coli* will be considered most fully, as there tended to be greater variation with this organism than with staphylococcus.

The procedure adopted was as follows: Ox serum was employed; the blood was obtained at the slaughter-house, and was received directly from the severed neck-vessels into a sterile bottle in which it was allowed to clot; the serum was pipetted off 24 to 48 hours later and was freed from red corpuscles by centrifuging; then it was heated for about an hour at 56° C. If the serum proved to be sterile it was measured in amounts of 1 c.c. into $3 \times \frac{1}{2}$ in. sterile test-tubes, which were plugged with cotton-wool; these were heated again at 56° C. for half an hour. In conducting a test varying dilutions of antiseptic in watery solution were added, the largest volume not exceeding 0·1 c.c.; then of a 24 hours' culture of the test organism in peptone water diluted with 0·85 per cent. saline 0·1 c.c. was added, the dilution usually employed being 1 : 1000, but the effect with other quantities of bacteria was also tested.

In general the following concentrations of antiseptic were employed: 1 : 1,000,000, 1 : 400,000, 1 : 200,000, 1 : 100,000, 1 : 40,000. The same strain of *B. coli* was used throughout; it was found that with different specimens of ox serum the amount of growth, as shown by the development of turbidity in the medium, varied considerably; hence inhibition of growth as judged by the eye was likely to be a less satisfactory criterion than sterility as determined by subculturing a loopful of the mixture on agar. The sterilising concentration is therefore taken as the lowest which failed to yield growth in subculture. Table I shows the results obtained in serum with the four specimens of acriflavine which have been most frequently examined.* The modal value, 1 : 100,000, which was obtained in 35 per cent. of the tests, corresponds with that originally assigned to this antiseptic. It is noteworthy that the efficiency of antiseptic action is practically independent of the inoculation doses used, the results with an inoculum of 0·001 c.c. being equal to those with 0·00005–0·000025 c.c. Additional experiments with product "P" on different occasions further emphasise this point (Table II). Thus while the magnitude of the inoculum increased from 1 to 100, the sterilising dose of the

TABLE I.—*Showing the Sterilising Concentration of Acriflavine for* B. coli *in Ox Serum (56° C.), as Determined in a Series of Sixty-eight Tests.*

Sterilising concentration.	Series A. Products.				Totals.	Series B. Products.		Totals.	Series C. Products.		Totals.	Sum of totals.
	O.	P.	Q.	R.		O.	P.		O.	P.		
1 : 1,000,000	1	—	—	4	**5**	—	2	**2**	—	1	**1**	**8**
1 : 400,000	—	1	1	7	**9**	—	1	**1**	—	7	**7**	**17**
1 : 200,000	1	—	—	7	**8**	4	—	**4**	1	3	**4**	**16**
1 : 100,000	4	—	3	5	**12**	3	—	**3**	8	1	**9**	**24**
1 : 40,000	—	—	2	—	**2**	—	—	—	—	—	—	**2**
< 1 : 40,000*	—	—	1	—	**1**	—	—	—	—	—	—	**1**
No. of tests					**37**			**10**			**21**	**68**

In series A the inoculum = 0·001 c.c. of culture per 1 c.c. of medium; series B = 0·00005 c.c; series C = 0·000025 c.c.

* This indicates that 1 : 40,000 failed to sterilise.

TABLE II.—*Showing the Sterilising Concentration of Acriflavine (Product P) for* B. coli, *as Determined with varying Inoculation Doses on Different Occasions.*

Medium = Ox serum 56° C.

Inoculum per 1 c.c. medium.	Sterilising concentration.
0·1 c.c.	1 : 100,000
0·01 ,, ...	1 : 130,000
0·002 ,, ...	1 : 200,000
0·001 ,, ...	1 : 400,000

---

* Product "O" is the original sample of "trypaflavin" given to us by Prof. Ehrlich.

antiseptic only varied from 1 to 4. In view of the general range of variation already noted with the same inoculation dose on various occasions, the orderly arrangement in this table is probably fortuitous. As regards the three experiments in which the sterilising dose was highest (1 : 40,000 or more), it is to be noted that on one of the occasions product " O " was tested simultaneously and the sterilising dose was found to be 1 : 100,000 ; thus there is no evidence that either the culture or the medium was abnormal on this occasion. The tests have extended over a period of more than four years and it is clear from the records that there is no grouping of the results with reference to time, *i. e.* there is no evidence in the case of *B. coli* that the organism has undergone any permanent or cyclic alteration in susceptibility. When the same product has been tested several times on the same day with the same specimen of serum and culture the results have shown a marked degree of uniformity. Thus product " R " was tested in five series, the only difference being that certain of them were shaken more frequently than others during the first few hours' incubation ; the result was that 1 : 1,000,000 sterilised in two series and 1 : 400,000 in three. Again, three specimens of product " P " (one being the specimen as originally furnished and the other two, A and B, prepared from it by recrystallisation) were tested on a single occasion, with the result that in two sterilisation was effected by a concentration of 1 : 400,000 and in the third by 1 : 200,000. As has been noted previously, different specimens of ox serum differ considerably in the luxuriance of growth which they yield when similarly inoculated with *B. coli*, but there appears to be no relationship between this factor and the sterilising concentration of antiseptic.

B. coli in peptone water.—The sterilising dose was originally stated to be 1 : 1300 ; at that time peptone water with a P_H value of less than 6·0 was employed, as noted above. The great intensification of antiseptic action which results when the P_H value of the medium is 7·2 to 7·8 is well shown in Table III. Thus the sterilising concentration under these conditions is 1 : 20,000.

TABLE III.—*Showing the Effect of Hydrogen-ion Concentration upon the Sterilising Concentration of Acriflavine for* B. coli *in Peptone Water in a Series of Tests.*

Inoculum = 0·001 c.c. culture per 1 c.c. medium.

	P_H of medium.	
Sterilising concentration.	Under 6·0.	7·2–7·8.
1 : 100,000	—	1
1 : 20,000	—	7
1 : 10,000	—	3
1 : 2,000	3	—
1 : 1,300	5	—
> 1 : 1,300*	8	—
1 : 1,000	3	—

* This indicates that 1 : 1300 failed to sterilise.

Staphylococcus.—Several strains of *Staphylococcus aureus* were employed at different times, but there was no evidence of any notable difference in their behaviour toward the antiseptic. As is shown in Table IV, the collected results confirm the originally assigned value of 1 : 200,000 as the sterilising concentration in serum (modal value obtained in 51 per cent. of the tests). In the case of peptone water medium, alteration in the hydrogen-ion concentration affects the values, but not to the same extent as occurs with *B. coli*. At P_H 7·2 to 7·8 the sterilising concentration lies in the neighbourhood of 1 : 200,000. It is striking that although serum is a much more suitable medium than peptone water, as shown by the abundance of growth, nevertheless the potency of the antiseptic is on the average identical in the two cases.

TABLE IV.—*Showing the Sterilising Concentration of Acriflavine for* Staphylococcus aureus *as determined in a Series of Tests.*

The inoculum per 1 c.c. of medium in the serum series consisted of 0·001 c.c. in 27 tests, 0·00005 c.c. in 15, 0·000025 c.c. in 19 and 0·000005 c.c. in 3: as no difference is attributable to this factor the results are grouped together. In the peptone water series 0·001 c.c. was used throughout.

Sterilising concentration.	Medium. Ox serum (56° C.). No. of tests.	Medium. Peptone water $P_H < 6·0$. No. of tests.	Medium. Peptone water P_H 7·2–7·8. No. of tests.
1 : 400,000	15	1	4
1 : 200,000	32	0	7
1 : 100,000	14	7	0
1 : 40,000	2	8	1
> 1 : 40,000*	0	1	0
Totals	63	17	12

*** This indicates that 1 : 40,000 failed to sterilise.**

THE ADDITION OF ANTISEPTIC TO THE MEDIUM AFTER GROWTH OF ORGANISMS HAS OCCURRED.

Experiments on the plan of those already described in serum might be considered to involve the fallacy that one is dealing with organisms which are not accommodated to the medium employed, since the inoculum is a peptone water culture. Accordingly the following observation was made: The serum was inoculated first of all, and after incubation for 48 hours at 37° C. varying amounts of acriflavine were added to the resulting growths. The effect was ascertained by making subcultures after a further period of incubation for 48 hours. The result was that the tubes containing the antiseptic in a concentration of 1 : 100,000 and upwards were rendered sterile. Therefore it is a striking fact that a heavy 48-hours' growth of organisms is killed with as great ease by the subsequent addition of the antiseptic as are a few organisms added simultaneously with the antiseptic. The experiment in which organisms are introduced first of all, and then the antiseptic is added after growth has occurred, resembles more closely than the others the condition met with in treating an infected wound. *B. coli* was employed in these tests.

REPEATED INOCULATION OF MEDIUM CONTAINING THE ANTISEPTIC.

The question as to whether the antiseptic tends readily to undergo deterioration in the serum or to become exhausted by the organisms appears to have been answered in the negative by previous observations (1, 2). Thus it was found that bactericidal action progresses for at least 24 to 48 hours at 37° C., and tubes which appear without visible growth at the end of this period do not subsequently show obvious proliferation. The point has been further investigated, however. A series of tubes containing serum with varying amounts of acriflavine were inoculated and kept at 37° C., the presence of viable colon bacilli being tested for by making subcultures on agar with one loopful of each mixture after 24 and 48 hours; at the end of this time they were re-inoculated with 0·001 c.c. of *B. coli* culture as usual. The results are shown in Table V. A similar series was re-inoculated after 96 hours; in this case the primary inoculation was sterilised in a concentration of 1 : 100,000, and 24 hours after re-inoculation the same tube yielded twelve colonies, while after 48 hours it was again sterile. Accordingly there is no evidence pointing to the inactivation of the antiseptic in the medium or to exhaustion by the organisms of the minute amounts which are active in producing bactericidal action.

TABLE V.—*Showing the Results of Reinoculation Experiments with Acriflavine and* B. coli *in Ox Serum* (56° *C.*).

Concentration of antiseptic.	Resulting growth from			
	Original inoculation after		Re-inoculation after	
	24 hours.	48 hours.	24 hours.	48 hours.
1 : 1,000,000	+	+	...	...
1 : 400,000	Slight inhibition	Inhibition	...	...
1 : 200,000	3 colonies	—	17 colonies	—
1 : 100,000	3 ,,	—	25 ,,	—
1 : 40,000	—	—	—	—

+ = abundant growth in subculture: — = no growth in subculture.

VARIABILITY OF RESULTS.

In seeking for an explanation of the variability in results met with in repeated tests (Tables I and IV), another phenomenon should be taken into account, viz. irregularity in sterilising action which may occur in a series of tubes containing ascending doses of antiseptic. Thus occasionally concentrations of 1 : 130,000 and 1 : 40,000 may sterilise the mixture, while 1 : 100,000 fails. This observation, which is more common with certain other substances of the acridine group, corresponds with that recorded by Richet and Cardot (6) on exaggerated variations in the amount of acid formed in different tubes of whey containing a constant concentration of certain antiseptics when inoculated similarly with a lactic organism. The assumption that a resistant strain of bacteria has been evolved suggests itself in view of the work of Ehrlich and his school on drug-resistance of trypanosomes, and of experiments on the acquired resistance to antiseptics of *V. choleræ* (Shiga[7]) and of the lactic organism (Richet and Cardot[6]). But in the present instance it must be emphasised that if such heightened resistance does in fact exist, it is probably of an inferior grade, and in our experiments, at least, the attempt to produce resistant strains both of *B. coli* and *Staphylococcus aureus* by continued subculture in peptone water medium containing acriflavine has so far been unsuccessful.([1]) Types of coliform bacilli which are highly insusceptible to the flavine compounds have been recovered occasionally from wounds treated with these substances (Drummond and McNee[5]), but the cultural characters of these organisms differ markedly from those of the common types of *B. coli* ([4]), and it appears most probable that their resistance, like that of *B. pyocyaneus*, is natural and not acquired. With regard to the cause of the irregularities we should incline to the following view: In the vicinity of the lethal concentration, especially with substances which destroy the life of the organisms only slowly, there is at first a fairly balanced equilibrium between, on the one hand, the tendency of the organisms to assimilate foodstuffs, and as a result to proliferate, and on the other, the inhibitory action of the antiseptic. Accordingly, it is probable that at this stage some relatively minute difference, which it is at present impossible to define precisely, may disturb the balance in favour of the organisms, thus leading to growth.

CONCLUSIONS.

(1) The sterilising concentrations of acriflavine (diaminoacridene methochloride) in heated ox serum (56° C.) for *B. coli* and *Staphylococcus aureus* are respectively 1 : 100,000 and 1 : 200,000, these being the modal values obtained in an extensive series of tests. These figures correspond with the values originally assigned.

(2) The sterilising concentrations in dilute peptone water, with a reaction P_H 7·2 to 7·8 (approximately), are found to be for *B. coli* 1 : 20,000, and for *Staphylococcus aureus* 1 : 200,000 (modal values). These values represent a much higher activity than those originally given; they are to be explained by the increased efficiency due to using medium of a lower hydrogen-ion concentration than that previously employed.

(3) The sterilising concentration of the antiseptic is within wide limits independent of the size of the inoculum.

(4) Different commercial specimens of acriflavine could not be distinguished as regards their antiseptic potency, but there was evidence that certain specimens were more irritating to the conjunctiva than others; this property is likely to be important where the substance is employed in treating an infection of a delicate membrane, *e. g.* that of the urethra.

(5) The occurrence of variations in the sterilising dose in repeated tests has been noted, and it appears that such differences are due to factors which cannot at present be completely defined.

(6) Methods of testing the antiseptic power of acriflavine by repeated

inoculation of a mixture of antiseptic and serum, and also by adding the antiseptic after the growth of organisms (*B. coli*) had occurred in the medium, have shown that rapid exhaustion or deterioration of the antiseptic in the medium does not occur. The latter experiment resembles more closely than the others the conditions met with in treating an infected wound. The striking fact has been elicited that a heavy growth of organisms is sterilised by the subsequent addition of the antiseptic with as great ease as are a few organisms added to the medium simultaneously with the antiseptic.

(7) Irregularity in the action of different concentrations of antiseptic in the same series has been observed, and the view has been advanced that, especially where the lethal effect is slowly produced, as with the compounds here considered, there is a critical concentration in the vicinity of which the balance may be inclined in favour of the organism or of the antiseptic by factors which at present escape complete analysis.

REFERENCES.

1. Browning, C. H., Gulbransen, R., Kennaway, E. L., and Thornton, L. H. D.—"Flavine and Brilliant Green: Powerful Antiseptics with Low Toxicity to the Tissues," *Brit. Med. J.*, 1917, **1**, 73.
2. Browning, C. H., Gulbransen, R., and Thornton, L. H. D.—"The Antiseptic Properties of Acriflavine and Proflavine, etc.," *ibid.*, 1917, **2**, 70.
3. Browning, C. H., Gulbransen, R., and Kennaway, E. L.—"Hydrogen-ion Concentration and Antiseptic Potency, with Special Reference to the Action of Acridine Compounds," *J. Pathol. and Bacteriol.*, 1919, **23**, 106.
4. Browning, C. H., and Gulbransen, R.—"The Testing of Antiseptics in Relation to their Use in Wound Treatment," *J. Hyg.*, 1919, **18**, 33.
5. Drummond, H., and McNee, J. W.—"On the Treatment of a Series of Recently Inflicted War Wounds with Flavine," *Lancet*, 1917, **2**, 640.
6. Richet, C., and Cardot, H.—"La transmission héréditaire des caractères acquis et l'accontumance des microbes," *Compt. rend. Acad. d. Sci.*, 1920, **171**, 1353; *Ann. de l'Inst. Pasteur*, 1919, **33**, 575.
7. Shiga, K.—"Über Gewöhnung der Bakterien an Farbstoffe," *Zeitschr. f. Immunitätsforsch.*, Orig., 1913, **18**, 65.

TECHNIQUE.

ON PICRO-CARMINE.

DR. VILH. JENSEN.

Institute for General Pathology, Copenhagen.

Received for publication January 22nd, 1921.

THE picro-carmine which Ranvier used for nearly all his staining work has been noted for its excellence for many years, but nobody else has been able to equal it, not even his pupils, many of whom have given formulæ for its preparation.

The reason may be that Ranvier worked with greater patience and slower methods than we do to-day, but chiefly, perhaps, because he always employed wet methods of staining and washing out under the cover-slip, where we now use paraffin or celloidin and mount in resin. It is also noteworthy that the only two workers that I have been able to find who recommend picro-carmine for staining, namely Stöhr and Mann, both advise the wet method and slow preparation after the manner of Ranvier.

In the course of time a number of different formulæ for picro-carmine have been given, most of which are mentioned in P. Mayer's excellent book on staining methods. The majority of them contain ammonia as a solvent for the carmine, but this makes their use impossible with paraffin sections, as the adhesive material used to fix them to the slide is dissolved and the section floats away.

From time to time I have tried many of these formulæ, as well as the liquid stains ready for use made by Grübler and Struer, but none of them were satisfactory.

If a solution of picric acid be gradually added to an alkaline solution of carmine a precipitate forms almost immediately, and the picric acid cannot, therefore, take an active part in the staining reaction. Moreover, the addition of magnesium picrate to magnesium-carmine, as mentioned by P. Mayer, does not give an adequate differential strain, because the picric acid, which functions as the differentiating medium, is wanting.

I therefore tried whether it was possible to combine both methods—the blending of a salt of picric acid with free picric acid—and after various attempts, by altering the constituents and the mode of mixing, I discovered the following:

1. Magnesium-carmine (P. Mayer): 1 grm. carmine and 0·1 grm. magnesium oxide are boiled in 50 c.c. distilled water for 5 minutes. Cool and filter. Then 0·5 c.c. liquid phenol is added.

2. Magnesium picrate (P. Mayer): 50 c.c. ½ per cent. picric acid solution in distilled water boiled for some minutes with 0·25 grm. magnesium oxide. Cool and filter.

3. 1 per cent. picric acid in distilled water.

Mix 1 and 2. Then add 10 c.c. of 3, shaking constantly.

This gives an absolutely clear, deep red staining fluid, which on account of the phenol present remains unaltered for months, perhaps years.

The stain applied for 3–5 minutes to a celloidin or paraffin section produces a beautifully differentiated effect, and the nucleus especially is nicely stained, the best results being obtained after hardening in alcohol or sublimate. The sections do not become detached from the slide when they are fixed thereon by the method described by Mann, which is the one used at the Institute.

In virtue of the excess of free picric acid this stain has the further advantage that it can be used instead of picric acid for the beautiful method of staining bacteria devised by Claudius, which is much quicker and more certain than Gram's method for sections. In addition to this the tissue is simultaneously stained, so that the usual preliminary staining with lithium-carmine, differentiation with acid alcohol and washing with water can be dispensed with, thereby considerably shortening the procedure.

Combined with the Claudius method of staining a paraffin section the rationale is as follows:

(1) Xylol, 1 min.; (2) wash with xylol; (3) wash with alcohol; (4) methyl-violet (1 per cent. watery solution), 2–3 min.; (5) wash with water; (6) picro-carmine, 3–5 min.; (7) pour off the stain and dry with thick filter-paper; (8) decolorise with picric-aniline (aniline with a trace of picric acid added) till it becomes light red; (9) wash with xylol; (10) dry with filter-paper; (11) xylol-damar, cover-glass.

Gram-positive bacteria are stained blue, nuclei red, protoplasm orange, muscle and blood yellow, and connective tissue a rose tint.

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

THE REACTION OF THE BLOOD IN SECONDARY ANÆMIA.

C. LOVATT EVANS.

From the Department of Pharmacology and Biochemistry, National Institute for Medical Research, Hampstead, London.

Received for publication March 16th, 1921.

THE reaction of blood, as is well known, is controlled by the presence in it of buffer substance. The buffering of separated plasma is due to the presence of sodium bicarbonate. Whole blood is more perfectly buffered than separated plasma, owing to a contribution of the red corpuscles. These different degrees of buffering are illustrated in the carbon dioxide reaction curves ($R\text{–}CO_2$ curves) shown in Fig. 1, which represents the change in reaction (P_H) with change in the carbon dioxide pressure of water or any dilute unbuffered solution (very approximately) of a separated plasma, a blood, and of a hypothetical perfectly buffered solution of neutral reaction. It will be seen that blood approaches more nearly to the perfectly buffered solution than does plasma, which nevertheless shows quite a good buffering power. The superior buffering of the blood is dependent on its hæmoglobin content; the greater this is, the more nearly does the reaction curve approach to that demanded of a perfectly buffered solution and *vice versâ*. The reasons for this will be discussed elsewhere. The buffering of

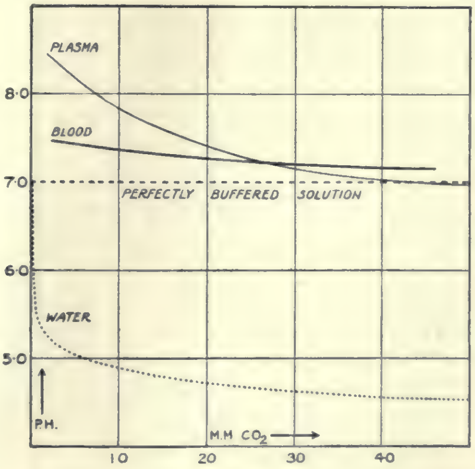


FIG. 1.

drawn plasma, which is not fundamentally different from that of any other aqueous solution, I have called *primary* or direct buffering. The better buffering of blood is due to what may be called *secondary* or indirect buffering, which is accomplished by the corpuscles. The corpuscles, by reason of an ionic interchange with the plasma (Hamburger) (1) virtually yield alkali (sodium bicarbonate) to the plasma, under all circumstances (*e. g.* dilution, addition of CO_2) which tend to render the blood more acid; the alkali is refunded again by a reversal of the same mechanism when there is any tendency for the blood to become more alkaline. The ratio $[NaHCO_3] : [CO_2]$, on which the reaction depends (L. J. Henderson) (2), is thus kept much more nearly constant in the plasma of whole blood than in separated plasma.*

The reaction of a blood is that of its plasma in contact with the corpuscles. Various methods have from time to time been employed for the determination of the hydrogen ion concentration of blood, but none of them are so simple and direct as the indicator method, a modified form of which was recently described by Dale and Evans.(3)

Since the reaction of blood depends on the ratio $NaHCO_3 : CO_2$ in its plasma, it is clear that when one of these is altered disproportionately to the other the reaction will change, whereas if both are altered in the same proportion no change in the reaction will take place. Accordingly we have distinctions drawn between "compensated acidosis" (lowering of bicarbonate, *i. e.* of alkali reserve, together with proportional reduction of CO_2), and "uncompensated acidosis" (a greater reduction in the alkali reserve than in the CO_2). A rise of CO_2 content with no change in the alkali reserve would also produce an "acidosis," as in cardiac dyspnœa, but of a different type. Increase in the ratio $NaHCO_3 : CO_2$ would cause a more alkaline blood, and this is often termed "alkalosis"; like an acidosis it can be compensated by simultaneous change of the CO_2 pressure. It is clear that the determination of the alkali reserve ($NaHCO_3$) of the plasma can by itself give no information as to the reaction of the blood, because it is impossible to say whether any change in the alkali reserve is compensated or not. But given two factors out of the three (P_H, CO_2 pressure, $NaHCO_3$ content, of the blood) we can get information regarding the third. Hasselbalch's method of calculation could be applied to find the P_H where the second and third factors (blood CO_2 and alkali reserve) are known, or the ratio of alveolar CO_2 to plasma CO_2 would itself give some measure of the acidity of the blood (Peters).(4) Since the estimation of the alveolar carbon dioxide pressure presents a good deal of difficulty it would appear to be simpler to determine the blood reaction directly, and if the alkali reserve is also determined it is possible to draw some inferences with regard to the carbon dioxide pressure of the circulating blood.

By also determining the corpuscular volume by the hæmatocrite and the oxygen capacity or hæmoglobin content, much information regarding the regulation of the reaction of the blood and the causes of changes in the reaction can be obtained. In any investigation on blood reaction all these data should, if possible, be obtained.

---

* A valuable account of the blood buffers has appeared while this paper was in the press: "The Carbon Dioxide Carriers of the Blood," by D. D. Van Slyke, *Physiological Reviews*, vol. i, p. 141.

These methods are applied in the present paper to the study of the effects of hæmorrhage on blood reaction.

METHODS.

Cats were bled, under general anæsthesia, from cannulæ in the carotid artery. A sample of the blood was run direct into one of the dialysers for the determination of the reaction as described by Dale and Evans (3) A second sample of blood was collected and mixed with a little solid potassium oxalate, beneath a layer of liquid paraffin contained in a centrifuge tube, centrifuged, and the plasma withdrawn for determination of the alkali reserve. A third sample of oxalated blood was collected for determination of the hæmoglobin content.

Rabbits were bled, without anæsthesia, from an ear vein. For the construction of the R–CO_2 curve portions of the blood were brought into equilibrium with air containing known pressures of CO_2, and samples of the equilibrated blood were used for determination of the P_H. Plasma was separated under liquid paraffin from the blood equilibrated with 42 mm. CO_2 (my own alveolar air).

Alkali reserves of blood and plasma were determined by the method of Van Slyke (5), oxygen capacity by Barcroft's method (6) and corpuscular volumes by the hæmatocrite.

THE IMMEDIATE EFFECTS OF HÆMORRHAGE.

Results obtained by several independent observers (*see* Medical Research Council (7), Milroy (8)) have shown that the alkali reserve of the blood-plasma is lowered immediately after a large hæmorrhage, and that the blood, at a given pressure of carbon dioxide, is then more acid than normal blood. The lowering of the alkali reserve after the withdrawal of moderate amounts of blood appears to be due principally to dilution of the blood with tissue fluids of lower bicarbonate content. After very severe hæmorrhage, however, another factor comes into play, viz. the production of fixed acids as a result of inadequate oxygen supply. Increased respiratory ventilation called forth by the increasing acidity of the blood may, within limits, compensate for the lowering of alkali reserve due to either or both of these causes, and may restore the blood to its original reaction; "air-hunger" therefore does not necessarily always mean the same thing as oxygen lack, though oxygen lack due to hæmorrhage is always associated with air-hunger. Whether the reaction of the circulating arterial blood in air-hunger following hæmorrhage is more acid than, or identical with, or more alkaline than that of normal blood depends on whether the increased ventilation by the lungs inadequately, exactly, or over-compensates the fall of alkali reserve, however this may have been produced. Experiments I and II illustrate cases of under- and over-compensation respectively. Whether the respiratory response under- or over-compensates for the change in alkali reserve apparently depends ultimately on whether the respiratory centre is respectively less or more excitable than before.

In Experiment I there was an uncompensated or only partially compensated acidosis.

EXPERIMENT I.—*Cat, 2300 grm. Under Urethane.*

Time.	Total amt. blood drawn.	Carotid B.P.	Arterial blood as drawn.		Hæmoglobin per cent.
			Alk. res. plasma.	P_H. of blood.	
12.11	5 c.c.	170	38	7·45	72
12.12	49 ,,	38	—	—	62
12.46	69 ,,	68	26	7·31	50

EXPERIMENT II.—*Cat, 2600 grm. Urethane.*

Time.	Total amt. blood drawn.	Carotid B.P.	Arterial blood as drawn.		Hæmoglobin per cent.
			Alk. res. plasma.	P_H. of blood.	
3.39	5 c.c.	148	44	7·25	90
3.57	61 ,,	110	33	7·38	68
3.58	Rapid shallow breathing sets in and lasts till 4.45, when it gets very slow.				
4.37	66 c.c.	72	27	7·38	—
4.45	Respiration becomes very slow.				
4.55	—	50	27	7·30	50
5.15	Respiration stops. Gave artificial respiration slowly.				
5.52	71 c.c.	32	16	7·15	50

In Experiment II, so long as the high rate of ventilation persisted, the blood after hæmorrhage was, in spite of the lowered alkali reserve of its plasma, less acid than that of the same (anæsthetised) cat previous to bleeding, but when the respiration became slow and the circulation also began to fail, an acidification, due apparently to oxygen lack, set in. Ultimately the respiration stopped and slow artificial respiration was given, but the circulation still continued to fail, and the alkali reserve of the plasma to fall; the blood then became very decidedly acid.

EXPERIMENT III.—*Cat, 2000 grm. Urethane. Cyanide Solution Injected into the Femoral Vein.*

Time.	B.P.	Arterial blood.			Remarks.
		Alkali reserve.	P_H.	Hæmoglobin per cent.	
12.26	104	40	7·35	80	Quiet respiration of normal anæsthetised animal.
1.8	64	26	7·16	74	One minute after last of six doses of 1 c.c. N/100 NaC.N. During the administration violent hyperpnœa followed by dyspnœa.

The effect of oxygen lack of the tissues in producing fixed acids which enter the blood is well seen in cyanide poisoning, in which the oxygen usages of the tissues is transitorily much depressed. The hyperpnœa seen in the early stages of the effect is, as I have shown (9), due to an effect of such acids on the respiratory centre. The effect on the reaction of the blood is shown by Experiment III.

In a similar experiment the blood after the administration of cyanide was found to contain 0·045 per cent. of lactic acid, which was separated in the form of well-shaped crystals of zinc lactate. Whereas the urine in the bladder at the commencement of the experiment had an acid-base ratio expressed by: 8·3 c.c. N/10 alkali to neutralise 10 c.c. to phenolphthalein : 9·4 c.c. N/10 acid to neutralise 10 c.c. to methyl orange = 0·88, that at the end was expressed n the same terms as 3·8 : 3·8 = 1·0, *i. e.* it was more dilute and more acid. The kidney had already commenced to eliminate more of the acid phosphate, and so to contribute to a restoration of the normal reaction of the blood.

The state of anæsthesia is not responsible in these experiments for the reduced alkali reserve of the blood after hæmorrhage, since blood drawn without anæsthesia shows the same lowering of alkali reserve.

EXPERIMENT IV.—*Rabbit, 2000 grm. Bled from Ear Vein; no Anæsthetic.*

Time.
11.30 Bled 25 c.c. alkali reserve of blood = 53 per cent. Alkali reserve of plasma separated from blood *in vitro* at 42 mm. CO_2 = 52 per cent.
12.30 Bled 15 c.c. alkali reserve of blood = 45 per cent. Alkali reserve of plasma *in vitro* at 42 mm. = 37 per cent.

THE REACTION OF THE BLOOD IN SECONDARY ANÆMIA.

The question of the duration of the acidosis resulting from hæmorrhage and of the form of the CO_2-reaction curve of the blood was investigated on rabbits, owing to the convenience with which these animals can be painlessly bled from the ear without the use of anæsthetics. It should be emphasised, however, that for such experiments on rabbits, constancy of the diet is of great importance. This was shown by Hasselbalch (10), and I have had abundant opportunity of confirming it. Lower alkali reserves are obtained with a diet of mangolds than with a mixed diet of oats, mangolds and bran. The mean alkali reserve of the blood of three rabbits fed on the mixed diet and kept out of doors was 63 per cent., when on the same diet and kept indoors 65 per cent., and when fed and kept indoors on mangolds (three days) 59 per cent. If for any reason an animal refuses to take food, the alkali reserve also goes down.

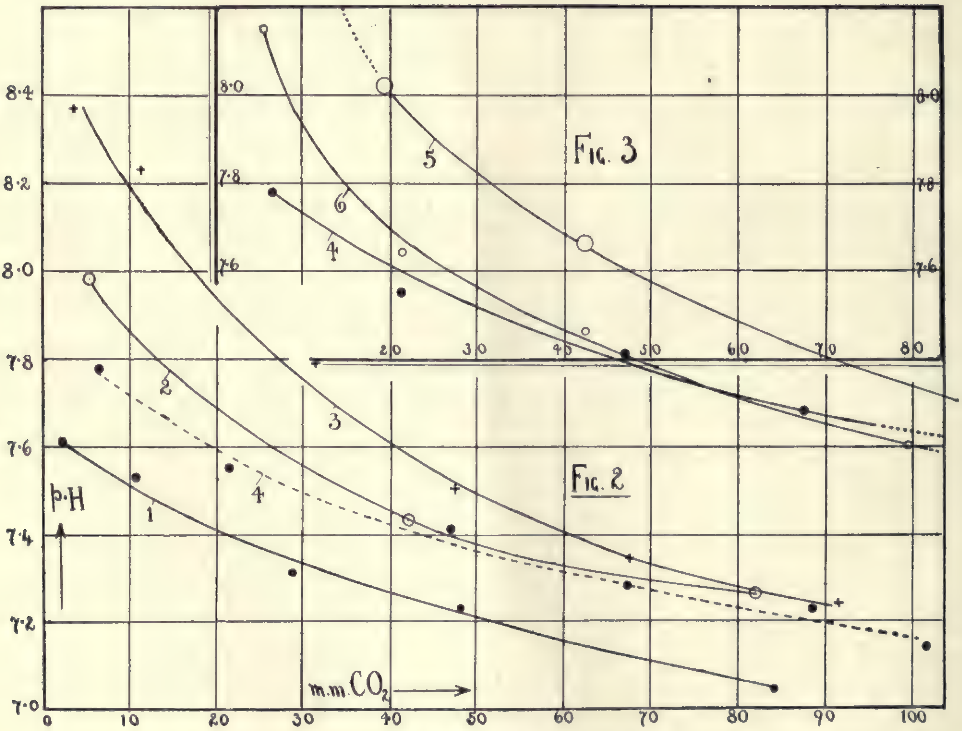
The CO_2-reaction curves were followed from time to time on one rabbit which was bled at intervals. Alkali reserves, oxygen capacities and corpuscular volumes were also determined on this rabbit, but in order to exclude irregular variations they were also observed on three other rabbits, on which also the reaction of the urine was followed. Figs. 2 and 3 give the results of the experiments on the R-CO_2 curves. The numbers on the curves refer to those of the sample numbers in the first column, Table I.

TABLE I.

EXPERIMENT 5.—*Rabbit, 2·3 kgrm. Blood Reaction Curves at 38° C. Zero day was the day of commencement of the experiment.*

No.	Day.	Blood sample. Volume.	Corpuscular volume.	O_2 capacity.	Alkali reserve blood.	Alkali reserve of plasma drawn at 42 mm. CO_2.
1	0	50 c.c.	26·5	10·7	38	33
2	1	20 ,,	17·9	7·7	63	60
3	2	40 ,,	14·6	5·8	52	61
4	18	50 ,,	33·7	11·4	51	51
5	19	40 ,,	18·5	8·2	51	61
6	21	55 ,,	12·6	5·3	42	44

It will be seen (Fig. 2) that the day after the first bleeding the blood is much more alkaline than it was to begin with, and this alkalinity is not lost even after eighteen days, by which time there has been an over-compensation both of corpuscular volume and oxygen capacity. Starting again at the eighteenth day (Fig. 3), it is seen that repetition of the bleeding does not lead to another rise, but eventually, when the bleeding had been rather excessive, to a definite fall. The increased slope of the reaction curves



FIGS. 2 AND 3.

shows the effect on the buffering power of the removal of corpuscles. Curve 4, Figs. 2 and 3, represents a buffering value at least equal to normal, though the whole curve is at a more alkaline level, but curves 2 and 3, as compared with 1 and 4, or 5 and especially 6, which, being nearer the same level, is more strictly comparable with 4, show that for a given rise of CO_2 pressure the P_H changes far more in the anæmic than in the normal blood. Thus, for a rise of CO_2 pressure from 30–50 mm., curves 1 and 4 show a fall in P_H of 0·12, whereas 2, 3, 5 and 6 show 0·17, 0·26, 0·24 and 0·20 respectively.

These abnormally steep curves would mean that the anæmic animal would be dyspnœic, or would become so on the slightest exertion, being abnormally liable to an acidosis, were it not for the fact that the alkali reserve of the whole blood is so raised as to put the curve above the possibility of such a thing happening. If, however, the reduction of the corpuscles is carried to such an extent that a lasting oxygen lack is established, then the alkali reserve of the blood is reduced again, and a vicious circle sets in.

The next experiment is one in which the recovery from a large hæmorrhage was traced at intervals during three weeks, at the end of which the effects of the hæmorrhage had been recovered from.

Experiment 6.—*Rabbit, 2 kgrm.*

Time from commencement.		Volume of blood taken.	Alkali reserve.		Corpuscular volume.	Hæmoglobin per cent.	Per cent. protein in plasma.	Per cent. chloride in plasma.
Days.	Hours.		Blood.	Plasma.				
0	0	50 c.c.	59	60	26·0	56	·655	·592
0	1½	10 ,,	56	52	18·2	35	·542	·600
0	5½	10 ,,	50	45	15·3	34	·548	·592
0	12	6 ,,	67	63	13·3	22	—	—
1	0	10 ,,	79	78	9·3	22	·548	·598
2	0	10 ,,	70	69	9·7	22	·586	·610
7	0	10 ,,	67	72	19·5	42	·605	·635
13	0	10 ,,	75	77	19·6	48	·602	·612
21	0	10 ,,	55	53	22·0	52	·655	·625
22	0	10 ,,	57	—	—	—	—	—

It is seen that the first fall in the alkali reserve of the plasma, which is associated with dilution of the blood, has passed off in twelve hours: thereafter the alkali reserve persists with fluctuations at a high level for two or three weeks. When the serum proteins, corpuscular volume and hæmoglobin content of the blood are restored to their original level, the alkali reserve also falls.

In order to exclude the irregular variations in the plasma alkali reserve in the rabbit an experiment was carried out simultaneously with three rabbits, the effect of variation of diet being also studied. The figures for acidity and alkalinity of the urine, which are also given, show the number of cubic centimetres of N/10 alkali or acid which would be needed to neutralise the day's urine to phenolphthalein or methyl orange. Figures for carbonate of urine, if any, represent the number of cubic centimetres of N/10 acid to neutralise the day's urine to phenolphthalein. Mean values for the three animals only are given, so that figures for both acid (*i.e.* for urines acid to phenolphthalein) and carbonate (*i.e.* urines alkaline to phenolphthalein) appear. Both of these are small, the significant titre being the alkalinity, to which, in the determination of the acid-base ratio, the carbonate figures were added.

Date.	Treatment.	Per cent. alkali reserve blood.	Twenty-four hours' urine.			Ratio, alk. + carb. : acid.
			Vol.	Acidity.	Alkalinity. + Carbonate.	
27.1.21	Out-door: mixed diet of mangolds, oats and bran	63	71	1·8	103 + 8·9	62
29.1.21	Indoor: ditto	65	151	2·2	116 + 2·5	54
2.2.21	Mangolds for three days indoors	59	260	3·0	261 + 1·5	87
3.3.21	26 hours after removal of 2·5 per cent. of body-weight of blood. Same diet.	69	168	5·3	80 + 0·0	15
4.2.21	Next day. Same diet.	68	218	28·0	185 + 0·0	6·6

The results show clearly that the increased alkali reserve of the blood is associated with, and possibly assisted or produced by, an increased output of acid by the kidneys. If the ammonia excreted in the urine had also been determined the total acid excretion as measured by acidity *plus* ammonia would probably have appeared even larger than that shown above (Hasselbalch (11), Fitz and Van Slyke (12)). A further contribution to the rise of alkali reserve of the blood might be made by a more complete absorption of the inorganic constituents of the food or by the taking of larger quantities of food, though there is good reason to believe that this was excluded in the above experiment. For a general account of the relations of the kidneys and lungs to blood reaction see Macleod.(13) Full references cannot be given here.

In an experiment on a cat the alkali reserve of the blood was 49 per cent.; of the plasma drawn from it at 42 mm. CO_2, 48 per cent.; twenty-four hours after withdrawal of 25 c.c. of blood the values were 52 per cent. and 50 per cent. respectively. Here also there is a slight, but definite, rise in the alkali reserve of the blood, and not, as is always the case immediately after the hæmorrhage, a fall.

The results would appear to have a definite biological significance. Increased alkalinity of the blood is, within limits, less likely to be attended with untoward results than is increased acidity. If the alkali reserve of the blood in secondary anæmia were the same as in the normal individual, any physical exertion sufficient to cause anoxæmia could easily lead to a definite acid reaction because of the greatly reduced buffering effect of the corpuscles; this is guarded against by an over-compensation of the alkali reserve. Probably these effects are more clearly seen in the rabbit than in many other animals or in man, because in the rabbit the respiratory centre is not sensitive enough to compensate for very small changes in the blood reaction.

Observations on the carbon dioxide content of human anæmic bloods, which possibly have a similar significance to those described above, have been made by Bieling (14) and by Barr and Peters.(15)

It is quite likely that the respiratory centre is set to respond to a different reaction of the blood in these cases where the alkali reserve of the blood is greatly altered. If this is so, then no exact conclusions as to the carbon dioxide pressure in the alveolar air or blood can be drawn from the R–CO_2 curve. That this is so is indicated by numerous clinical cases where there is a lack of correspondence between alveolar CO_2 and plasma bicarbonate, without there being any evidence that the respiratory centre is unduly active or the reverse.

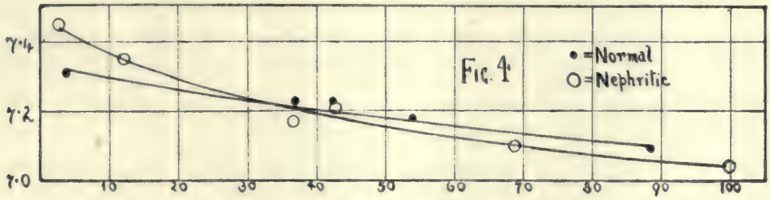


FIG. 4.

What really seems to be of the greatest importance is the avoidance of too great an acidity. The breathlessness of anæmic subjects is explicable from the steep slope of the R–CO_2 curve. Exercise, especially if severe enough to give rise to anoxæmia and so to the production of fixed acids, produces a more rapid change in the blood reaction, and the respiratory centre responds vigorously, not because the blood is now more acid than, or even as acid as, a normal resting blood, but because the centre is now tuned up to maintain a reaction well on the alkaline side of the normal. The mechanism by which the excitability of the respiratory centre is modified is probably the oxygen tension of the blood by which it is supplied (Hasselbalch (10)).

The response of the blood reaction in anæmia is apparently different from that in other states in which the blood reaction is liable to alteration. Fig. 4 shows a comparison of normal blood with that from the same animal rendered nephritic by the subcutaneous injection of uranium nitrate sixty hours previously. The anæmic blood shows a reduced buffering power as judged by the slope of the curve, but the alkali reserve of the blood, very low to begin with, shows no change in the direction of compensation.

CONCLUSIONS.

Although the immediate effect of hæmorrhage is a lowering of the alkali reserve of the blood, the response of the respiratory centres may partially compensate, or even over-compensate, for this change, so that the circulating blood may be either more acid, or less acid, or of the same reaction even after as before the hæmorrhage. After twelve hours the reduction in alkali reserve is compensated, owing to increased excretion of acid by the kidney; subsequently the alkali reserve of the blood may become higher than the normal and remain at this level until the corpuscular regeneration is complete. The response is a protective one against acidosis.

REFERENCES.

1. Hamburger, H. J.—"Ionenwanderungen in Serum und Blut under den Einfluss von CO_2, Säure und Alkali," *Biochem. Ztschr.*, 1917, **86**, 309.
2. Henderson, L. J.—"Das Gleichgewicht zwischen Basen and Säuren im tierischen Organismus," *Ergebn. d. Physiol.*, 1909, **8**, 254.
3. Dale, H. H., and Evans, C. L.—"Colorimetric Determination of the Reaction of Blood by Dialysis," *J. Physiol.*, 1920, **54**, 167.
4. Peters, J. P.—"Carbon Dioxide Acidosis, the Cause of Cardiac Dyspnœa," *Amer. J. Physiol.*, 1917, **43**, 113. "Respiratory Response to Rapid Changes in Blood Reaction," *ibid.*, **44**, 84.
5. Van Slyke, D. D.—"A Method for the Determination of Carbon Dioxide and Carbonates in Solution," *J. Biol. Chem.*, 1917, **30**, 347.
6. Barcroft, J.—'The Respiratory Functions of the Blood,' Cambridge, 1914.
7. M. R. C.—'Acidosis and Shock,' Special Report Series No. 25, vii, 1918.
8. Milroy, T. H.—"The Reaction Regulator Mechanism of the Blood before and after Hæmorrhage," *J. Physiol.*, 1917, **51**, 259.
9. Evans, C. L.—"Observations on Cyanide Anoxæmia," *ibid.*, 1919, **53**, 17.
10. Hasselbalch, K. A.—"Neutralitätsregulation und Reizbarkeit des Atemzentrums in ihren Wirkungen auf die Kohlensäurespannung des Blutes," *Biochem. Ztschr.*, 1912, **46**, 403.
11. *Idem.*—"Ammoniak als physiologischer Neutralitätsregulator," *ibid.*, 1916, **74**, 18.
12. Fitz, R., and Van Slyke, D. D.—"The Relationship between Alkali Reserve and Acid Excretion," *J. Biol. Chem.*, 1917, **30**, 389.
13. Macleod, J. J. R.—"The Diagnosis of Acidosis," *J. Lab. Clin. Med.*, 1919, **4**, 315.
14. Bieling, R.—"Experimentelle Untersuchungen über die Sauerstoffversorgung bei Anämien," *Biochem. Ztschr.*, 1914, **60**, 421.
15. Barr, D. P., and Peters, J. P., Jun.—"The Carbon Dioxide Absorption Curve and Carbon Dioxide Tension of the Blood in Severe Anæmia," *J. Biol. Chem.*, 1920, **45**, 571.

THE MECHANISM OF AUTOLYSIS IN PAROXYSMAL HÆMOGLOBINURIA.

WARRINGTON YORKE AND J. W. S. MACFIE.

From the Liverpool School of Tropical Medicine.

Received for publication February 15th, 1921.

HAVING had a case of paroxysmal hæmoglobinuria under observation for about eighteen months, we have taken the opportunity to re-examine the mechanism of the autolysis which produces the attacks of hæmoglobinuria, with the object of ascertaining, first, whether the reaction, shown by Eason, and Donath and Landsteiner to occur *in vitro*, suffices to explain the production *in vivo* of hæmoglobinæmia and hæmoglobinuria, and secondly, of extending the work of these authors, and of finding, if possible, a solution of certain observations such as the absence of hæmolysis after prolonged exposure of red cells and serum at 0° C., for which so far as we are aware no satisfactory explanation has hitherto been furnished.

In our earlier experiments we repeated and, in the main, confirmed the *in vitro* observations of Eason ([1]), Donath and Landsteiner ([2, 3]) and Widal and Rostaine ([4, 5, 6]). We also repeated the classical *in vivo* experiments of Ehrlich. As a result of these preliminary observations, we were greatly impressed with the fact that the amount of hæmolysis obtained in the *in vivo* experiments was about ten times as great as that obtained in the *in vitro* experiments with whole blood, or with a mixture of serum and red cells in the proportion in which they are found in the circulation.

With the object of obtaining a solution of this anomaly experiments were undertaken to determine, firstly, the shortest period it was necessary to keep the mixture of serum and erythrocytes at 0° C., and, secondly, the minimum extent to which it was necessary to lower the temperature of the mixture, in order to obtain the maximum degree of hæmolysis *in vitro* (Tables I, II and III).

As a result of the two series of experiments outlined in Tables I, II and III, it follows that in order to obtain the greatest degree of hæmolysis *in vitro* it is necessary to reduce the mixture of serum and erythrocytes to at least 5° C. for at least six or seven minutes. These conditions were, however, satisfied both in our *in vivo* experiments and also in our *in vitro* experiments with whole blood or with mixtures of serum and erythrocytes in the proportion in

TABLES I AND II.—*Showing the Result of Subjecting Mixtures of Hæmoglobinuric Serum and Erythrocytes to a Temperature of 0° C. for Various Periods.*

Table I: A weak suspension of erythrocytes was used.

Patient's serum.	Patient's red cells, 1 per cent. suspension in ·9 per cent. NaCl.	Time mixtures kept at 0° C.	Result after 1 hour at 37° C. and centrifuging.	
			Deposit.	Supernatant fluid.
c.c.	c.c.	Minutes.		
1·0	1·0	·5	Full	*Nil*
1·0	1·0	1	Almost full	Very light red
1·0	1·0	2	$\frac{3}{4}$	Light red
1·0	1·0	3	$\frac{2}{3}$	Red
1·0	1·0	4	About $\frac{1}{2}$	,,
1·0	1·0	5	About $\frac{1}{3}$	,,
1·0	1·0	60	*Nil*	,,

Table II: A concentrated suspension of erythrocytes was used.

Patient's serum.	Patient's red cells, concentrated suspension.	Time mixtures kept at 0° C.	Result after 1 hour at 37° C. and centrifuging.	
			Deposit.	Supernatant fluid.
c.c.	c.c.	Minutes.		
1·0	·04	1	Almost full	Faint red
1·0	·04	4	$\frac{1}{2}$	Red
1·0	·04	7	A little	Deep red
1·0	·04	10	,,	,,
1·0	·04	15	,,	,,
1·0	·04	60	,,	,,

TABLE III.—*Showing the Result of Cooling the Mixture of Hæmoglobinuric Serum and Erythrocytes to various temperatures.*

Hæmoglobinuric serum.	Patient's red cells, 1 per cent. suspension.	·9 per cent. saline.	Result after keeping at the following temperatures for 1 hour and then at 37° C. for 1 hour. Degree of hæmolysis.			
c.c.	c.c.	c.c.	15° C.	10° C.	5° C.	0° C.
1·0	1·0	0	Trace?	Complete	Complete	Complete
·75	1·0	·25	*Nil*	$\frac{1}{2}$	,,	,,
·5	1·0	·5	*Nil*	Trace	$\frac{3}{4}$	Almost complete
·25	1·0	·75	*Nil*	*Nil*	Trace	Slight
·1	1·0	·9	*Nil*	*Nil*	*Nil*	*Nil*

which they exist in the circulating blood. Why then did so much more hæmolysis take place *in vivo* (3–4 per cent.*) than *in vitro* (0·3 per cent.)? Whilst casting round for an explanation it occurred to us that possibly the fact that in the *in vitro* experiments the blood was kept at 0° C. for one hour, whereas in the *in vivo* experiments the finger was immersed in ice-water for five minutes only, might account for the discrepancy. Investigation proved this actually to be the case: when in later *in vitro* experiments the blood was exposed to a temperature of 0° C. for five minutes only, instead of for one hour, hæmolysis took place equal in amount to that occurring *in vivo* in the finger-immersion experiments, viz. 3–4 per cent.

We were therefore confronted with the remarkable paradox that incubation at 37° C. for one hour of whole blood (whether oxalated, defibrinated, or allowed to clot) previously exposed to a temperature of 0° C. for a period of sixty minutes resulted in but comparatively little hæmolysis (0·3 per cent.), whereas when the exposure to 0° C. was for five minutes only, hæmolysis of at least ten times the amount was obtained. With the object of obtaining more precise information on this subject two experiments were performed.

Experiment 4.—In the first experiment, blood (Table 4 A) withdrawn from the median basilic vein was put immediately into a series of tubes standing on ice (about 0·5 c.c. into each tube); the various tubes were left on ice for periods varying from half a minute to twenty-four hours and then were placed in a water-bath at 37° C. for one hour, the amount of hæmoglobin in the serum being consequently determined. In the second experiment (Table IV B), instead of whole blood a mixture of serum 6 volumes + erythrocytes 4 volumes (this being approximately the proportion in which the liquor sanguinis and erythrocytes were present in the circulating blood) was employed and the experiment continued as in A.

The results of these experiments showed clearly that the amount of hæmolysis occurring varied in a striking manner with the length of time the blood was kept at 0° C. The percentage of hæmoglobin free in the serum rose rapidly until a maximum was obtained after an exposure to cold of from 5 to 7·5 minutes. When the cooling was prolonged beyond this limit the amount of hæmolysis occurring rapidly diminished, until after 30 minutes only 10–15 per cent. of the maximum hæmolysis was obtained. It will be observed, however, that even after 24 hours at 0° C. some hæmolysis did take place, and that the quantity was greater in the case of the mixture of serum (6 parts) and red cells (4 parts) than in that of the whole blood which was allowed to clot. Similar experiments in which the blood or mixture of serum and red cells was kept at 0° C. for 24 hours amply confirmed this; sometimes the amount of hæmolysis obtained was distinctly less than in Experiment IV, but in the case of the mixture of serum and red cells it was always greater than in the case of the clotted blood.

These observations confirm in the main Donath's and Landsteiner's statement that 12 hours' exposure of hæmoglobinuric blood at 0° C. was not followed by hæmolysis when the blood was subsequently placed in the incubator at 37° C. As in practically all the experiments recorded in the papers of these authors and in those of Widal and Rostaine the time of exposure of the blood at 0° C. was at least half an hour, it would appear that these workers failed to realise what an important factor the time of exposure at 0° C. was in the reaction.

---

* The amount of hæmoglobin contained in the serum or plasma was determined with the aid of a Zeiss comparison spectroscope as described by Barratt and Yorke ([8]). It should be understood that when we state that serum or plasma contains a certain percentage of hæmoglobin we mean that there is in it as much hæmoglobin as in a solution containing a similar percentage of normal human erythrocytes.

TABLE IV.—*Showing how Time of Exposure of Blood, or of a Mixture of Serum 6 vols. and Erythrocytes 4 vols., to Cold (at 0° C.) influences the Degree of Hæmolysis which results after subsequent exposure to 37° C. for one hour.*

Tube.	Time left on ice at 0–1° C.	Time left in water-bath at 37° C.	Percentage of hæmoglobin free in serum.	
	Minutes.	Minutes.	A In experiment performed with whole blood allowed to clot.	B In experiment performed with serum 6 vols., erythrocytes 4 vols.
1	0	60	·1	·3
2	·5	60	2·4	—
3	1·0	60	5·0	3·6
4	3·0	60	6·0	5·75
5	5·0	60	8·25	6·75
6	7·5	60	8·25	6·75
7	10·0	60	8·0	5·0
8	12·5	60	3·0	4·5
9	15·0	60	2·5	4·0
10	20·0	60	1·2	—
11	30·0	60	·8	1·1
12	45·0	60	·5	—
13	60·0	60	·35	·75
14	90·0	60	·4	—
15	120·0	60	·275	1·1
16	150·0	60	·225	—
17	240·0	60	·275	—
18	300·0	60	·275	—
19	24 hours	60	·45	1·0

WHY IS PROLONGED COOLING NOT FOLLOWED BY HÆMOLYSIS?

Whereas Donath and Landsteiner, in explanation of their observation that when the exposure of hæmoglobinuric blood at 0° C. was prolonged to 12 hours hæmolysis did not subsequently take place at 37° C., merely stated that a further change must have occurred after the absorption of the lysin by the erythrocytes, Widal and Rostaine considered the phenomenon to afford strong support to their "insufficiency of antisensibilisatrice" theory. As, however, Widal and Rostaine's interpretation appears to depend on hypothesis and not on experimental evidence, it can hardly be regarded as convincing, and consequently the following experiments were devised with the object of investigating the explanation of the paradox.

Experiment 5.—January 12th, 1919: Blood was withdrawn from median basilic vein: some of it was put into a solution consisting of 1 per cent. sodium chloride in distilled water, and the rest allowed to clot at 37° C. for a couple of hours, the serum then being removed from the clot. The oxalated blood was immediately centrifuged, and the red cells, after removal of the oxalated plasma, washed thrice with normal saline; finally a 1 per cent. suspension of the washed red cells was made in normal saline.

Hæmoglobinuric serum (4 c.c.) was then added to hæmoglobinuric erythrocytes (1 c.c.), and the mixture allowed to stand 24 hours at 0° C., as were also the rest of the hæmoglobinuric serum and the red cells and those from the normal individual.

January 13th, 1919.—Serum and washed erythrocytes were again obtained from the hæmoglobinuric patient, and from the normal individual, in the usual manner. The mixture of serum (4 c.c.) and red cells (1 c.c.) which had stood all night on ice was centrifuged, the serum decanted, and the red cells washed thrice in iced saline solution. This serum is designated "treated" serum and the red cells "treated" red cells.

The following mixtures were then made, and after standing 5 minutes on ice, were placed in a water-bath at 37° C. for 1 hour; the degree of hæmolysis was then determined. The results are shown in Table V:

TABLE V.

	Serum 4 volumes.	Red cells 1 volume.	Result after keeping 5 minutes at 0° C., and 1 hour at 37° C. Percentage of hæmoglobin.
1	"Treated"	"Treated"	·18
2	,,	Hæmoglobinuric kept on ice for 24 hours	·14
3	,,	Hæmoglobinuric fresh	·08
4	,,	Normal individual fresh	·08
5	Hæmoglobinuric kept at 0° C. for 24 hours	"Treated"	3·9
6	Ditto	Hæmoglobinuric kept at 0° C. for 24 hours	3·9
7	,,	Hæmoglobinuric fresh	3·6
8	,,	Normal fresh	4·5
9	Hæmoglobinuric fresh	"Treated"	6·0
10	,,	Hæmoglobinuric kept at 0° C. for 24 hours	5·4
11	,,	Hæmoglobinuric fresh	6·5
12	,,	Normal fresh	6·3
13	Normal human kept at 0° C. for 24 hours	"Treated"	4·5
14	Ditto	Hæmoglobinuric kept at 0° C. for 24 hours	·44
15	,,	Hæmoglobinuric fresh	·32
16	,,	Normal fresh	·1
17	Normal human fresh	"Treated"	5·7
18	,,	Hæmoglobinuric kept at 0° C. for 24 hours	·4
19	,,	Hæmoglobinuric fresh	·2
20	,,	Normal fresh	·06

This experiment shows:

(1) That merely allowing hæmoglobinuric serum to stand (in the absence of erythrocytes) at 0° C. for 24 hours lessens its auto- and iso-lytic action, but only to a comparatively slight degree.

(2) That normal human serum which has stood (in the absence of erythrocytes) at 0° C. for 24 hours produces slightly less hæmolysis when added to erythrocytes which had been in contact with hæmoglobinuric serum for 24 hours than does fresh normal human serum.

(3) That hæmoglobinuric serum which has been in contact with hæmoglobinuric erythrocytes for 24 hours at 0° C. loses its power to hæmolyse the erythrocytes either of the hæmoglobinuric himself or of a normal individual.

(4) That hæmoglobinuric erythrocytes which have been in contact with hæmoglobinuric serum for 24 hours at 0° C. and then washed thrice with iced saline are loaded with lysin, as hæmolysis occurs not only when they are treated with fresh hæmoglobinuric serum, but also when they are treated with normal human serum. It will be observed that this is at variance with the statement by Widal and Rostaine, that red cells which have been in contact with hæmoglobinuric serum for 12 hours have lost the power of being hæmolysed by their own or normal human serum.

It is clear from Experiment 5 that the comparatively slight degree of autolysis which results when such mixtures of hæmoglobinuric serum and erythocytes are placed in a water-bath at 37° C. after a 24 hours' sojourn at 0° C. cannot be explained on the assumption that the red cells have failed to absorb immune body, since when normal human serum is added to red cells which have previously been in contact with hæmoglobinuric serum for 24 hours at 0° C. a considerable degree of hæmolysis results. It would rather appear that the absence of autolysis in such cases must depend on a deficiency of complement in the serum (after 24 hours' contact with red cells at 0° C.) at the time the mixture was put into the water-bath at 37° C. In order to examine these questions more fully the following experiment was devised:

Experiment 6.—Serum and washed erythrocytes were prepared from the hæmoglobinuric patient and from a normal individual in the manner already described (Exp. 5). Mixture A consisted of hæmoglobinuric serum (6 vols.) + hæmoglobinuric erythrocytes (4 vols.). Mixture B consisted of hæmoglobinuric serum (19 vols.) + hæmoglobinuric erythrocytes (1 vol.). These mixtures were placed in a number of tubes and allowed to stand at 0° C. for periods varying from 5 minutes to 24 hours, as shown in Table VI; they were then centrifuged and the serum examined for hæmoglobin and for the presence of autolysin (immune body) and complement. The test for immune body was carried out by adding to 4 vols. of the various sera 1 vol. of hæmoglobinuric erythrocytes, the mixture, after standing at 0° C. for 5 minutes, being centrifuged on ice and the serum removed; normal human serum (4 vols.) was then added to the red-cell deposit as complement, and after incubation at 37° C. for 1 hour the degree of hæmolysis determined (Table VI). In order to test for complement, 1 volume of a 5 per cent. suspension of sheep's red-cells, sensitised by the addition of the requisite quantity of inactivated immune rabbit serum, was added to various quantities of the sera, and the smallest quantity of serum necessary to produce complete hæmolysis in 1 hour at 37° C. ascertained.

The results obtained from this experiment may be summarised as follows:

(1) The decrease in hæmolysis which results after prolonged contact of red cells and serum at 0° C. is greater in A than in B—*i. e.* is greater where the concentration of red cells is greater.

TABLE VI.—*Showing the Amount of Hæmolysis which resulted after allowing Mixtures A and B to stand at 0° C. for various periods; and also the Results of Examining for Autolysin and Complement the Sera after they have been in contact with red cells at 0°C. for varying periods.*

Tube.	Mixture.	Time mixtures kept at 0° C.	Degree of hæmolysis resulting.	Result of examination of sera for—		
				Immune body (test carried out as described in text). Percentage of hæmoglobin.	Complement.	
					Smallest volume of serum required to lake completely 1 volume of 5 per cent. sensitised sheep's red cells.	Relative amount of complement present in various sera.
Control hb serum.		—	—	6·6	·12	100
1	A	5 mins.	0	—	1·0	12·0
2	A	5 mins. + 60 mins. at 37° C.	6·6	—	10 vols. gave no hæmolysis	0
3	A	10 mins.	0	2·8	2·0	6·0
4	A	15 mins.	0	·72	4·0	3·0
5	A	30 mins.	0	·24	10 vols. gave no hæmolysis	0
6	A	60 mins.	0	—	Ditto	0
7	A	2 hours	0	—	,,	0
8	A	2 hours + 60 mins. at 37° C.	·6	—	,,	0
9	A	24 hours	·2	—	,,	0
10	A	24 hours + 60 mins. at 37° C.	·36	—	,,	0
11	B	5 mins.	0	—	·2	60·0
12	B	5 mins. + 60 mins. at 37° C.	3·3	—	10 vols. gave no hæmolysis	0
13	B	10 mins.	0	—	·3	40·0
14	B	15 mins.	0	—	·4	30·0
15	B	30 mins.	0	6·0	1·0	12·0
16	B	60 mins.	0	—	10 vols. gave slight hæmolysis	·3
17	B	2 hours	0	—	10 vols. gave no hæmolysis	0
18	B	2 hours + 60 mins. at 37° C.	2·1	—	Ditto	0
19	B	24 hours	·24	6·0	,,	0
20	B	24 hours + 60 mins. at 37° C.	·65	—	,,	0

(2) In A, which contains an excess of red cells, immune body is constantly removed from the serum until after 30 minutes it has almost disappeared; in B, which contains a relatively small concentration of red cells, only a small fraction of the immune body is removed after 30 minutes, and the fact that this subsequently undergoes no further decrease suggests that the red cells must be saturated with immune body after 30 minutes.

(3) In A, complement disappears from the serum with great rapidity, only 12 per cent. being left after 5 minutes, 3 per cent. after 15 minutes, and 0 per cent. after 30 minutes; in B the disappearance of complement is slower, 60 per cent. being left after 5 minutes, 30 per cent. after 15 minutes, and 12 per cent. after 30 minutes.*

(4) The fact that in B only about 10 per cent. of immune body is removed from the serum, whereas all the complement is removed, indicates that immune body is present in the hæmoglobinuric serum in amount greatly in excess of the complement.

(5) Notwithstanding complete or almost complete disappearance of immune body and complement from the serum in contact with red cells at 0° C., no hæmolysis takes place at this temperature. After cooling for 24 hours slight hæmolysis was, however, observed in both A and B, even before incubation at 37° C.; very little more was obtained after subsequent incubation at 37° C. for one hour.

The general conclusion from this experiment appears to be, therefore, that even though immune body and complement are rapidly absorbed from hæmoglobinuric serum by erythrocytes at 0° C., hæmolysis does not take place, or does so only with extreme slowness, and to a very slight extent, at this temperature.

With a view to obtaining further information on the subject the following experiment was performed:

Experiment 7.—Serum and washed erythrocytes were prepared from the hæmoglobinuric patient in the manner already described, and a series of mixtures of serum and red-cells made as follows:

Tubes A, B, C, D and X, each containing serum ·6 c.c., erythrocytes ·03 c.c.; tubes E, F and Y, each containing serum ·6 c.c., erythrocytes ·4 c.c.; tubes X and Y kept at 0° C. for five minutes and then at 37° C. for one hour and the degree of hæmolysis determined; tubes A, B, C, D, E and F kept at 0° C. for one hour, then Tubes A and F kept at 37° C. for one hour and the degree of hæmolysis determined; tubes B, C, D and E centrifuged on ice, the serum removed and the erythrocytes washed thrice in iced saline.

Serum B was tested for complement, which was found to be absent, 20 vols. failing to hæmolyse 1 vol. of a 3 per cent. suspension of sensitised sheep's erythrocytes in 1 hour at 37° C. The mixtures in Table VII were then made and kept at 37° C. for 1 hour and the degree of hæmolysis determined. (*Vide* Table VII.)

The contents of Tube D were now shaken up and divided into two equal parts, D 1 and D 2. D 1 was kept at 0° C. for 5 minutes and D 2 at the laboratory temperature for a similar period; they were then centrifuged and the saline replaced by an equal volume of normal human serum, incubated at 37° C. for 1 hour. On centrifugalisation the supernatant fluid in each tube was found to be red.

The results obtained in Experiment 7 can be summarised as follows:

(1) Although no complement is detectable in serum which has stood in

---

* Experiments showed that there was no deficiency of complement in the hæmoglobinuric serum as compared with normal human serum when the two were tested against sensitised sheep's corpuscles.

contact with erythrocytes at 0° C. for one hour, yet it does contain something which is essential for the production of hæmolysis when the mixture is heated to 37° C. Apparently, therefore, the complement is split into its two portions, mid- and end-piece, the mid-piece* uniting at 0° C. with the sensitised red cells, and the end-piece only uniting with the combination sensitised red cells + mid-piece (persensitised red cells) on subsequently warming to 37° C.

(2) As has been shown in previous experiments, when the proportion of serum and red cells are the same as those in the circulating blood, viz. 6 to 4, then the amount of hæmolysis which results when the cooling is for one hour is relatively slight as compared with that obtained when the cooling is for five minutes only (1·0 per cent. as compared with 6·6 per cent.). When, however, the proportion of serum and erythrocytes is 20 to 1, the amount of hæmolysis is practically the same whether the cooling be for five minutes or for one hour (3·3 per cent. in each case).

TABLE VII.

Tube.	Washed erythrocytes.	Serum.	Percentage of hæmoglobin in the serum after keeping at 37° C. for one hour.
1	B	C	3·0
2	C	E	3·3
3	D	Saline	0·0
Tube X			3·3
„ Y			6·6
„ A			3·3
„ F			1·0

These observations taken in conjunction with the results obtained in the previous experiments appear to us to warrant the following inferences:

(1) Immune body is present in hæmoglobinuric serum in large amount; it unites with red cells at 0° C., and if the volume of serum is constant, the number of red cells sensitised will, within limits, depend on the number of red cells added, *e. g.* in Experiment 6 A many more red cells are sensitised than in Experiment 6 B.

(2) Mid-piece unites with sensitised red cells at 0° C. On comparing Experiments 6 A and 6 B it is seen that all the mid-piece is removed from the serum in each case, although in Experiment 6 A rather more rapidly than in

---

* Browning and Watson (9) have previously recorded the fact that when fresh hæmoglobinuric serum was treated with an equal volume of washed human red blood-corpuscles at 0° C. for 1½ hours certain constituents of the complement were removed from the serum along with the immune body by the red corpuscles; as the result of further experiments they reached the conclusion that the constituent of the complement fixed at 0° C. was not identical with the globulin fraction (mid-piece) obtained by treatment of fresh normal serum with carbonic acid gas.

Experiment 6 B. This fact can only mean that the concentration of mid-piece attached to each sensitised red cell in Experiment 6 A is much less than in Experiment 6 B, as in the latter case the number of sensitised red cells is much fewer than in the former.

(3) On warming to 37° C. end-piece unites with the mid-piece immune body red-cell combination (*i. e.* with persensitised red cells); consequently, the concentration of end-piece acting on each red cell will depend entirely on the concentration of mid-piece or, in other words, on the number of red cells present.

(4) Assuming that a definite concentration of end-piece (*e. g.* at least two units for each red cell) is necessary for the production of hæmolysis, it is clear that when the red cells are present in great excess as in Experiment 6 A (serum 6 vols., red cells 4 vols., *i. e.* the proportion of serum and red cells of the circulating blood), the necessary concentration of end-piece will only be found on occasional red cells and hence but slight hæmolysis is seen, and furthermore, that when the red cells are present in relatively small numbers only, as in Experiment 6 B (serum 6 vols., red cells 0·03 vols.), the necessary concentration of end-piece will be found on many red cells and consequently much hæmolysis results.

(5) Although the above hypothesis may afford a satisfactory explanation why cooling for one hour at 0° C. does not result in appreciably less hæmolysis than does cooling for five minutes in cases where the concentration of red cells is low as in Experiment 6 B, and why the reverse is the case in cases where the concentration of red cells is high (Experiment 6 A), yet it does not explain the whole of the phenomenon. Reference to Experiment 6 shows that even in the case where the concentration of red cells is low, less hæmolysis is obtained after cooling at 0° C. for two hours than after cooling for five minutes, and that after twenty-four hours' cooling there is relatively slight hæmolysis. This fact suggests that after dissociation of complement into its two components, degeneration of one or both fractions gradually takes place.

In light of information obtained from Experiments 6 and 7 and from a number of similar experiments, we are now in a position to offer some explanation of the curious paradox that comparatively little hæmolysis results after hæmoglobinuric blood (or a mixture of hæmoglobinuric serum 6 vols., and erythrocytes 4 vols.) has stood at 0° C. for half an hour or more as compared with that which occurs when the sojourn at 0° C. is for five minutes only.

When hæmoglobinuric blood is placed on ice, immune body is rapidly absorbed by the erythrocytes until it is completely removed from the serum (Experiment 6 A). Complement is dissociated into its two fractions, mid-piece and end-piece, the mid-piece uniting with the sensitised erythrocytes. Immune body is, however, present in hæmoglobinuric serum in amount greatly in excess of complement. The result is that when hæmoglobinuric blood has stood at 0° C. for thirty minutes or more, all the immune body is united with the erythrocytes and all the complement is dissociated into its two fractions, the mid-piece being diffused over a relatively very large number of sensitised red cells. The hæmolysis which results when the blood is now transferred to the incubator at 37° C. is but slight for the following reasons ;

(1) Hæmolysis is entirely dependent on the reunion of the dissociated components of the complement.

(2) The amount of end-piece which can take effect on any sensitised red cell is limited by the amount of mid-piece attached to that cell; and owing to the great diffusion of mid-piece amongst a relatively enormous number of sensitised red cells, it is usually so small as to be insufficient to provoke lysis.

(3) Degeneration of one or both fractions of the dissociated complement gradually takes place, and consequently the longer hæmoglobinuric blood is kept at O° C., the less, within limits, is the hæmolysis which results on subsequently warming to 37° C.

When, however, hæmoglobinuric blood is kept at 0° C. for but 5 minutes a portion only of the immune body is removed from the serum, and consequently fewer red cells are sensitised; moreover, the complement is not completely dissociated into its two components, about 12 per cent. of the amount orginally present being still recognisable in the serum. The hæmolysis which results when the blood is now transferred from the ice-chest to the incubator is relatively great for the following reasons:

(1) Hæmolysis is not entirely dependent on reunion of the two dissociated components of the complement, as a certain amount (about 12 per cent.) of undissociated complement is still available to exert its hæmolytic effect.

(2) As the number of sensitised red cells is fewer, the amount of mid-piece attached to each sensitised red cell is greater, and consequently, on warming to 37° C., the amount of end-piece which can take effect on each sensitised red cell is also greater.

(3) The exposure at 0° C. being of short duration, very little or no degeneration of the fractions of the dissociated complement takes place.

This hypothesis appears to afford, in our opinion, a satisfactory explanation of the results obtained by us in our various experiments—results which at first sight appeared anomalous or even contradictory.

REVERSIBILITY OF THE ERYTHROCYTE-IMMUNE BODY REACTION.

In view of the fact that erythrocytes (hæmoglobinuric or normal human) absorb immune body from hæmoglobinuric serum at low temperatures only, the question arises whether the reaction is "reversible." In other words, will raising the temperature of the sensitised red cells cause the union of erythrocytes and immune body to disintegrate? It has already been shown by Landsteiner (10) that this occurs in the case of auto-agglutinin present in the blood of many normal animals, and by Yorke (11) in that of auto-agglutinin found in the blood of man and animals infected with trypanosomes. The question of the reversibility of the erythrocyte-immune body reaction was considered by Donath and Landsteiner (2) in their original paper, but apparently they arrived at no definite conclusion, as they merely state that if it does occur it must do so with extreme slowness.

Browning and Watson (9) studied the question of dissociation of immune body at 37° C. in their two cases of paroxysmal hæmoglobinuria. Dissociation was obtained in one case, but not in the other, and in the first case only when the corpuscles used were those of the case itself, and not when they were those of a normal individual. We have examined the point in the following experiments:

Experiment 8.—Hæmoglobinuric serum and washed erythrocytes and normal human serum and washed erythrocytes were prepared in the usual way. Into each of five tubes A–E were put equal volumes of a mixture containing hæmoglobinuric serum (4 vols.) and hæmoglobinuric erythrocytes (1 vol.). The tubes were allowed to stand on ice for 5 minutes. Tube A was then placed immediately into a water-bath at 37° C.; intense hæmolysis occurred, the percentage of hæmoglobin free in the serum being 4·8. Tubes B, C, D and E were centrifuged on ice, the sera removed and the red deposits washed thrice with iced saline. To each of the deposits 4 volumes of iced saline were then added, and Tubes B and C allowed to stand at 37° C. for half an hour, and Tubes D and E at 0° C. for a similar time. At the end of the half hour Tubes B and C were centrifuged at 37° C., and Tubes D and E at 0° C., and the supernatant salines (digest solutions) removed. The red-cell deposits B and D were then examined for immune body by the addition of 4 volumes of normal human serum, and incubating at 37° C. for 1 hour; deposits C and E were used as controls (Table VIII A). Digest solutions B and D were examined for the presence of immune body by adding to each 1 volume of fresh hæmoglobinuric erythrocytes, allowing to stand at 0° C. for 5 minutes, centrifuging on ice, removing the digest solution, and adding to the red cells 4 volumes of normal human serum and incubating at 37° C. for 1 hour; digest solutions C and E were used as controls (Table VIII B).

TABLE VIII A.

Tube.	Red-cell deposit. 1 vol.	Normal human serum or saline. 4 vols.	Result after incubating at 37° C. for 1 hour. Percentage of hæmoglobin in the serum or saline.
1	B	Normal human serum	·8
2	C	Saline	·06
3	D	Normal human serum	6·3
4	E	Saline	0

TABLE VIII B.

Tube.	Digest solution. 4 vols.	Fresh hæmoglobinuric red cells.	Result after standing 5 mins. at 0° C., centrifuging on ice, removing digest solutions, adding to red cells 4 vols. of normal human serum or saline and incubating at 37° C. for 1 hour.
1	B	1 vol.	Normal serum, 2·7
2	C	,,	Saline, ·3
3	D	,,	Normal serum, ·04
4	E	,,	Saline, ·02

The results obtained in this experiment show that the erythrocyte-immune body reaction is reversible, the erythrocytes absorbing immune body at 0° C., and disgorging it at 37° C.

The question was now examined whether the substance present in the digest solution was pure immune body or a combination of this with mid-piece.

Experiment 9.—Three tubes, A, B and C, each containing hæmoglobinuric serum 4 vols. + hæmoglobinuric erythrocytes 1 vol., were kept at 0° C. for half an hour; they were then centrifuged, the serum decanted and the red cells washed thrice in iced saline. Red cells A, B and C were then digested at 37° C. in 4 vols. of saline for half an hour; the digest solution was removed by centrifugalisation and the red cells washed thrice in saline at 37° C. Examination of extracted Serum B showed that it contained practically no complement, 4 vols. of the serum causing only slight hæmolysis when added to 1 vol. of a 1 per cent. suspension of sensitised sheep's erythrocytes. The digested red cells, the digest solutions and the extracted sera were then examined for immune body and mid-piece and end-piece, as is shown in the Tables IX A and IX B.

TABLE IX A.—*Showing Result of Examination of Digested Red Cells for Immune Body and Mid-piece and End-piece.*

Red-cell deposit. 1 vol.	Serum or saline. 4 vols.	Result after incubation at 37° C. for 1 hour. Percentage of hæmoglobin in the serum or saline.
A	Normal human serum	3·2
B	Extracted Serum A	2·2
C	Saline	0

The results show that the digested red cells contained immune body + mid-piece, but no active end-piece.

TABLE IX B.—*Showing Result of Examination of the Digest Solutions for Immune Body, Mid-piece and End-piece.*

Digest solutions A, B and C (4 vols.) were each added to fresh hæmoglobinuric red cells (1 vol.) and kept at 0° C. for 5 minutes; then after centrifuging on ice the treated red cells were washed thrice with iced saline.

Treated red cells.	Serum or saline.	Result after incubating at 37° C. for 1 hour. Percentage of hæmoglobin in serum or saline.
A	Normal human serum	2·8
B	Extracted serum A	0
C	Saline	0

The results show that the digest solution contained immune body, but no mid-piece.

In the following experiment the question whether the immune body + mid-piece combination became dissociated from the erythrocytes at 37° C. was examined in a somewhat different manner.

Experiment 10.—Two tubes, A and B, each containing hæmoglobinuric serum 4 vols. + hæmoglobinuric erythrocytes 1 vol., were kept at 0° C. At the end of one hour A was centrifuged, the serum removed and the erythrocytes washed thrice in iced saline; normal human serum (4 vols.) was then added and the mixture kept at 0° C. for one hour, when it was again centrifuged, the serum removed, and an additional quantity (4 vols.) of normal human serum added. This procedure was repeated until complement (mid-piece) was no longer removed from the normal serum, as shown by its hæmolytic effect on sensitised sheep's red cells or, in other words, until the sensitised hæmoglobinuric erythrocytes were completely saturated with mid-piece and failed to take up any more. This condition was not reached until the process of adding normal human serum had been repeated six times. When hæmoglobinuric erythrocytes A were completely saturated with mid-piece (6½ hours after the commencement of the experiment), they were washed thrice in iced saline, as were also hæmoglobinuric erythrocytes B which had been standing in contact with hæmoglobinuric serum during the whole period.

Hæmoglobinuric erythrocytes A and B (1 vol.) were now digested in saline (4 vols.) at 37° C. for half an hour, and the erythrocytes and digest solutions examined for immune body and mid-piece in the manner described in the previous experiment (Tables X A and X B.)

TABLE X A.—*Showing Result of Examination of Digested Red Cells for Immune Body and Mid-piece.*

Red-cell deposit, 1 vol.	Serum. 4 vols.	Result after incubation at 37° C. for 1 hour. Percentage of hæmoglobin in the serum.
A	Normal human	1·0
A	Extracted B	·64
B	Normal human	1·2

TABLE X B.—*Showing Result of Examination of Digest Solutions for Immune Body and Mid-piece.*

Treated red cells.	Serum.	Result after incubation at 37° C. for 1 hour. Percentage of hæmoglobin in serum.
A	Normal human	0
A	Extracted B	0
B	Normal human	·72

This experiment shows that the immune body mid-piece combination is not disgorged by the red cells on warming to 37° C.

IS THE AUTOLYSIN THERMOSTABLE OR THERMOLABILE?

It will be remembered that with regard to this subject different results were obtained by Donath and Landsteiner on the one hand and by Widal and Rostaine on the other. Two cases examined by Browning and Watson illustrate the variability in the thermostability of the hæmoglobinuric immune body in different cases; after heating at 55° C. for half an hour the serum of one case still contained immune body, whilst that of the other possessed practically no sensitising action.

Experiment 11.—Serum and washed erythrocytes were obtained from the hæmoglobinuric patient and a normal individual in the manner already described. A portion of the hæmoglobinuric serum was heated to 55° to 56° C. for half an hour. The following mixtures were then made and cooled at 0° C. for 5 minutes:

A. Heated hæmoglobinuric serum (4 vols.) + hæmoglobinuric erythrocytes (1 vol.).
B. Heated hæmoglobinuric serum (4 vols.) + normal human erythrocytes (1 vol.).
C. Unheated hæmoglobinuric serum (4 vols.) + hæmoglobinuric erythrocytes (1 vol.).
D. Unheated hæmoglobinuric serum (4 vols.). Normal human erythrocytes (1 vol.).

The mixtures were then centrifuged on ice, the serum decanted and the red-cell deposits washed thrice in iced saline. To each of the red-cell deposits 4 vols. of fresh normal human serum was then added, the mixtures incubated at 37° C. for 1 hour and afterwards centrifuged and the percentage of the hæmoglobin in the serum determined (Table XI).

TABLE XI.—*Showing the Effect on Different Occasions of Heating Autolysin to 55° to 56°C. for half an hour.*

Tube.	Erythrocyte deposit. 1 vol.	Normal human serum.	Result after incubation at 37° C. for 1 hour. Percentage of hæmoglobin serum. Observations made on—				
			Jan. 18th.	Jan. 24th.	Feb. 24th.	May 15th.	May 16th.
1	A	4 vols.	*Nil*	*Nil*	Deep red	1·6	3·0
2	B	,,	—	—	—	4·8	5·1
3	C	,,	6	Deep red	Deep red	5·1	5·1
4	D	,,	—	—	—	6·9	7·2

The results obtained in experiments of this kind were contradictory: those obtained on January 18th and 24th showed the autolysin to be thermolabile, whereas those obtained on February 24th, and on May 15th and 16th, showed it to be thermostable. We are, at present, unable to offer any explanation of these apparent discrepancies, but the general conclusion seems to be that heating to 55° to 56° C. for half an hour destroys, in part, at least, the autolysin and isolysin in hæmoglobinuric serum.

Similar experiments performed with "digest solution" (*i. e.* the solution obtained by extracting sensitised red cells with normal saline at 37° C.) showed the auto- and isolysin to be thermostable in every instance.

CONCLUSIONS.

(1) In these experiments we have confirmed the work of Donath and Landsteiner, Widal and Rostaine, Eason and others, showing that cooling to 0° C. the blood of a case of paroxysmal hæmoglobinuria, or a mixture of his serum and erythrocytes, is followed by hæmolysis on subsequently warming to 37° C.

(2) The amount of hæmolysis resulting from exposure *in vitro* of hæmoglobinuric blood at 0° C. for 1 hour is only about 10 per cent. of that obtained *in vivo* by immersing a ligatured finger in ice for 5 minutes. The explanation of this is found to be in the fact that the period of exposure at 0° C. in the two instances was different, viz. 60 minutes and 5 minutes respectively, this factor profoundly affecting the degree of hæmolysis.

(3) The greatest degree of hæmolysis *in vitro* was obtained when the hæmoglobinuric blood was cooled to 0° C. for 5 to 7·5 minutes, and was about 10 times as great as that resulting after exposure at 0° C. for half to one hour. It is possibly this fact which explains the difficulty experienced by Zinsser (12) and others in confirming Donath and Landsteiner's observations, in whose recorded experiments, as also in those of Widal and Rostaine, the time of exposure of the hæmoglobinuric blood at 0° C. was at least half an hour.

(4) The explanation of the paradox is to be found—

(i) In the behaviour of the complement, which consists apparently of two distinct elements, mid-piece and end-piece, the former of which unites with sensitised red cells at 0° C., whilst the latter unites with the persensitised red cells only at higher temperatures (37° C.).

(ii) In the fact that in hæmoglobinuric serum immune body is greatly in excess of complement, although there is no deficiency of the latter as compared with normal blood. Many more red cells are sensitised after an hour's exposure of hæmoglobinuric blood at 0° C. than after 5 minutes' exposure, and consequently mid-piece is diffused amongst a correspondingly greater number of sensitised cells; on warming to 37° C. end-piece unites with the persensitised red cells, the concentration available for each persensitised red cell depending directly on the number of the latter; when this is very great, as in the case of blood cooled at 0° C. for half an hour or more, the concentration is insufficient to produce lysis.

(5) The immune body red-cell reaction is reversible, union taking place at 0° C., and the combination dissociating at 37° C. The reaction ceases to be reversible so soon as mid-piece has united with the sensitised red cells, or, in other words, as soon as the red corpuscles have become persensitised.

(6) Experiments conducted with the object of determining the effect of heat on the autolysin were inconclusive; on two occasions the immune body was apparently destroyed by heating to 56° C. for half an hour, whilst on three other occasions its action was only slightly impaired.

REFERENCES.

1. Eason, J.—"The Pathology of Paroxysmal Hæmoglobinuria," *J. Pathol. and Bacteriol.*, 1906, **11**, 167.
2. Donath, J., and Landsteiner, K.—"Ueber paroxysmale Hämoglobinurie," *München. med. Wochenschr.*, 1904, **51**, 1590.
3. *Idem.*—"Weitere Beobachtungen über paroxysmale Hämoglobinurie," *Centralbl. f. Bakteriol.*, Orig., 1908, **45**, 205.
4. Widal and Rostaine.—"Insuffisance d'antisensibélisatrice dans le sang des hémoglobinuriques," *Compt. rend. Soc. de biol.*, 1905, **58**, 321.
5. *Idem.*—"Insuffisance d'antisensibélisatrice dans le sang d'un hémoglobinurique (Interpretation)," *ibid.*, 1905, **58**, 372.
6. *Idem.*—"Sérotherapie préventive de l'attaque d'hémoglobinurie paroxystique," *ibid.*, 1905, **58**, 397.
7. Ehrlich, P.—"Paroxysmale Hämoglobinurie," *Deutsche med. Wochenschr.*, 1881, **7**, 224.
8. Barratt, J. O. W., and Yorke, W.—"An Investigation into the Mechanism of Production of Blackwater," *Ann. Trop. Med. and Parasitol.*, 1909, **3**, 1.
9. Browning, C. H., and Watson, H. F.—"Paroxysmal Hæmoglobinuria," *J. Pathol. and Bacteriol.*, 1912, **17**, 117.
10. Landsteiner, K.—"Ueber Beziehungen zwischen dem Blutserum und den Korperzellen," *München. med. Wochenschr.*, 1903, **50**, 1812.
11. Yorke, W.—"Auto-agglutination of Red Blood-cells in Trypanosomiasis," *Proc. R. Soc.*, ser. B, 1911, **83**, 238.
12. Zinsser.—'Infection and Resistance,' New York, 1918 (Macmillan & Co.), p. 147.

ON THE ANTIGENIC PROPERTIES OF ACETONE-EXTRACTED BACTERIA.

S. R. DOUGLAS, M.R.C.S., Capt. I.M.S. (ret.),

Acting Director, Bacteriological Department, National Institute for Medical Research, Hampstead, London,

AND

ALEXANDER FLEMING, F.R.C.S.,

Assistant Director, Inoculation Department, St. Mary's Hospital, London.

Received for publication May 3rd, 1921.

When a bacterial vaccine is injected into man or other animals it is probable that the first stage in the production of the immunity which follows the injection is a process of solution of the bacteria. This is necessary in order that the antigenic constituents of the bacteria may come into sufficiently close contact with the cells and body fluids to permit of the production of the recognised antibodies.

When carrying out a series of experiments it was noted that if bacteria, such as *B. typhosus*, *B. dysenteriæ*, or Morgan's bacillus, had been thoroughly extracted with acetone, by means of a Soxhlet's apparatus, they became very much more soluble in tryptic and other proteolytic ferments. Further, bacilli thus treated were in a very convenient form for storage, namely a sterile, dry, white powder, which apparently kept its properties unchanged indefinitely. It was, therefore, decided to carry out a series of experiments to ascertain if vaccines prepared from bacilli which had been thoroughly extracted with acetone had equal antigenic properties to those which, for instance, had been made by sterilising suspensions in salt solution at the lowest necessary temperature, and to investigate in other ways the properties of bacilli thus treated. The work was commenced in 1916, but it was interrupted by other and more urgent war work, and, in consequence, many experiments which suggested themselves could not be carried out. The results, however, are sufficiently interesting to be recorded, and form a preliminary to work which is proceeding.

METHOD OF PREPARATION OF THE ACETONE-EXTRACTED VACCINE.

The bacteria were grown on agar in Roux bottles for twenty-four to forty-eight hours, and were washed off with a minimum quantity of salt solution.

This suspension was enumerated in the ordinary way against red blood-cells, and a small portion was sterilised by heat in the manner usual in the preparation of vaccines. This small portion was used as a control vaccine. The greater part of the suspension was measured and poured into an excess of acetone. The bacteria were precipitated by the acetone, and settled to the bottom as a flocculent mass, leaving the supernatant fluid clear. After twenty-four hours the clear supernatant fluid was decanted, and the whole mass of the precipitate was placed in a Soxhlet apparatus and extracted with acetone for about forty hours. The solid mass was then collected from the thimble and allowed to dry, protected from contamination, at room temperature, or in the incubator at 37° C. The amount of the dried mass was then obtained by weighing, and by a comparison with the count which had been made of the portion retained for the control vaccine and the original bulk of the suspension the number of bacteria per milligram could be estimated. In this way, from the same cultures, vaccines containing the same number of microbes could be prepared, consisting of heated and of acetone extracted bacteria. It was found that 1 mgrm. of acetone-extracted typhoid bacilli contained 3900 million bacilli, while the same weight of acetone extracted staphylococci represented 4800 million cocci.

MICROSCOPICAL CHARACTERS OF THE BACTERIA AFTER EXTRACTION WITH ACETONE.

Typhoid bacilli appeared as clear-cut Gram-negative bacilli.

B. welchii (twenty-four hours' growth) showed clear-cut bacilli, for the most part Gram-negative, but there were many which retained Gram's stain. There was a marked difference in the reaction to Gram's stain between the living culture and the acetone-extracted bacilli.

Staphylococci showed little change from their normal appearance. There were only a few cocci which did not retain Gram's stain.

Streptococci were almost wholly Gram-negative. Their form was preserved.

Gonococci showed no change.

EFFECT OF TRYPTIC FERMENTS ON ACETONE-EXTRACTED BACTERIA.

The trypsin used in these experiments was Allen & Hanbury's *Injectio trypsini co.* The organisms employed were *B. typhosus*, *B. welchii*, *Staphylococcus*, *Streptococcus*, *Gonococcus* and *B. dysenteriæ*, Shiga.

Serial dilutions were made of a solution of trypsin, and to 1 c.c. of each of these dilutions was added 1 c.c. of a suspension of acetone-extracted bacteria in a strength sufficient to give a very definite opacity to the mixture. Controls were put up of the same suspension of bacteria with salt solution instead of dilutions of trypsin, and also of trypsin dilutions and living bacteria. These controls in every case remained unchanged. The tubes were incubated for several hours at 50° C., and the results in respect of the acetone-extracted bacilli were as follows:

> Typhoid bacilli were almost completely dissolved in all dilutions of trypsin up to 1 in 2560 (the highest dilution employed) in half an hour.

Staphylococci were apparently not dissolved, but they were strongly agglutinated, even in the higher dilutions of trypsin, this agglutination being marked up to a dilution of 1 in 100,000 of trypsin.

B. welchii was dissolved by trypsin up to a dilution of 1 in 5000.

B. dysenteriæ and *Gonococci* were dissolved by the same dilutions of trypsin as *B. typhosus*.

A COMPARISON OF THE ACETONE-EXTRACTED BACILLI AND HEATED BACILLI AS REGARDS THE YIELD OF ANTIBODIES FOLLOWING THEIR INJECTION INTO ANIMALS.

Vaccines of the acetone-extracted bacteria and heated bacteria from the same culture were made of the same numerical strength. The same number of the extracted and unextracted bacteria were injected into rabbits, and the antibody contents of the sera of these rabbits were tested usually every day. The same numerical dose was chosen, as it was desired to ascertain whether there had been any destruction in the antigenic power of the bacteria in the process of extraction with acetone. It is possible that some of the antigenic substance was destroyed, but what remained, being in a more soluble form, gave the same result as a larger quantity contained in the unextracted bacteria, but in this research a simple comparison of numerically equal doses only has been made. Most of the work was carried out with the typhoid bacillus, as it is easier to test the antibodies of this organism than it is of others, such as *B. welchii*. The results obtained are, for the most part, set forth in tabular form.

B. typhosus.

Two series of rabbits were inoculated with the ordinary typhoid vaccine and with acetone-extracted vaccine. The same numerical doses were given in each case. Two rabbits in each experiment were inoculated with each of the vaccines. The agglutinating, bactericidal power and opsonic powers were, as a rule, tested daily, and the results are set forth in the form of tables.

In the first experiment one of the rabbits inoculated with the heated vaccine died ten days after the second dose of typhoid vaccine (the death of the rabbit had apparently nothing to do with the vaccine).

(*a*) *Agglutination*.—It will be seen from Tables I and II that, whereas the first injection of 500 millions subcutaneously resulted in a greater production of agglutinins when the ordinary vaccine was used, the second injection of 1000 millions, also subcutaneously, gave the advantage to the acetone-extracted vaccine. In the second experiment, where the subcutaneous doses were followed by two intravenous inoculations, the ultimate result was exactly the same in all four rabbits, so that their serum agglutinated the bacillus in a dilution of 1 in 51,200. So far as the agglutinins go, therefore, there did not seem to be any difference between the antigenic properties of the two vaccines.

(*b*) *Bactericidal power*.—This was tested in two ways:

(i) By the method described by Wright, in which volumes of undiluted serum are mixed with similar amounts of varying dilutions of a twenty-four hours old broth culture.

TABLE I.—*Agglutination of* B. typhosus *following the injection of heated and acetone-extracted vaccines. Injections given subcutaneously*, 500 *millions on April* 17*th, and* 1000 *millions on April* 27*th. The same emulsion, a formolised broth culture, was employed throughout the experiments. Readings were made after* 1 *hour at* 50° *C. The figures in the tables represent the highest dilution giving standard agglutination (Dreyer).*

	Heated vaccine.		Acetone-extracted vaccine.	
Date.	Rabbit No 1.	Rabbit No. 2.	Rabbit No. 3.	Rabbit No. 4.
April 17	Inoculated with 500 million bacilli subcutaneously.			
,, 25	400	400	200	200
,, 26	400	400	200	100
,, 27	800	400	400	200
,, 27	Inoculated with 1000 million bacilli subcutaneously.			
,, 28	800	200	400	200
,, 29	400	200	200	100
May 1	400	800	800	3,200
,, 2	400	800	1,600	3,200
,, 3	800	800	3,200	6,400
,, 4	800	1,600	3,200	12,800
,, 5	800	1,600	3,200	12,800
,, 6	Died	1,600	3,200	—
,, 7	—	800	3,200	6,400
,, 10	—	800	3,200	6,400
,, 11	—	800	3,200	3,200
,, 12	—	400	3,200	3,200

TABLE II.—*Agglutination of* B. typhosus *following the injection of heated and acetone-extracted vaccines. Injections of* 500 *millions and* 1000 *millions given subcutaneously on May* 2*nd and May* 12*th respectively, while* 1000 *millions given on May* 24*th and June* 13*th were administered intravenously. Technique as in the former experiment.*

	Heated vaccine.		Acetone-extracted vaccine.	
Date.	Rabbit No 1.	Rabbit No. 2.	Rabbit No. 3.	Rabbit No. 4.
May 2	0	0	0	0
,, 2	Inoculated with 500 million bacilli subcutaneously.			
,, 5	5	80	80	40
,, 6	40	800	200	100
,, 7	400	1,600	800	400
,, 8	800	3,200	800	800
,, 9	800	1,600	400	400
,, 10	800	800	400	400
,, 11	800	800	400	200
,, 12	400	400	200	200
,, 12	Inoculated with 1000 million bacilli subcutaneously.			
,, 13	200	400	400	200
,, 15	400	1,600	800	800
,, 16	400	1,600	1,600	1,600
,, 17 ,, 18 ,, 19	800	1,600	1,600	3,200
,, 20	800	1,600	1,600	1,600
,, 22	800	1,600	1,600	3,200
,, 24	Inoculated with 1000 million bacilli intravenously.			
,, 25	400	1,600	1,600	3,200
,, 26	400	800	1,600	3,200
,, 27	800	1,600	3,200	6,400
,, 29 ,, 30 ,, 31	12,800	6,400	3,200	12,800
June 5 ,, 10	12,800	12,800	3,200	12,800
,, 13	12,800	12,800	3,200	25,600
,, 13	Inoculated with 10,000 million bacilli intravenously.			
,, 14	6,400	6,400	1,600	6,400
,, 24	51,200	51,200	51,200	51,200

(ii) By a method which is described as follows:

Into a series of dwarf test-tubes, similar in size to those used by one of us (A. F.) for carrying out the Wassermann reaction with minimal quantities of serum, 40 c.mm. of saline solution are placed, with the exception of the first, in which is placed 40 c.mm. of undiluted serum. To the second tube of the series 40 c.mm. of serum is added, giving 80 c.mm. of a 1 in 2 dilution of the serum. After mixing, 40 c.mm. of the 1 in 2 dilution is removed from the second tube and placed in the third tube, making a mixture of 1 in 4 of the serum. This process is continued along the series of tubes, 40 c.mm. of the dilution contained in the last tube being discarded. As a result each tube of the series contains 40 c.mm. of serum varying in strength from undiluted, in the first tube, to 1 in 128 in the eighth tube, each one of the series being half the dilution of the one next to it. Having made these dilutions, 40 c.mm. of a 1 in 100,000 dilution of a twenty-four hour broth culture of *B. typhosus* is added to each tube. After thorough mixing has been obtained by tilting the tubes several times, the battery of tubes is sealed with plasticine and incubated for two hours at 37° C., so that the bactericidal power of the serum may be allowed to act. To ascertain if all the bacilli have been killed by any given dilution of the serum a volume of broth containing 1 per cent. mannitol, and an indicator (the volume employed was 100 c.mm.) is added to each tube, and the whole series is again sealed with plasticene and incubated at 37° C. overnight. On examination next day any tube in which the bacilli have been killed off will show no change in colour, while those which contained living bacilli will be recognised by the change in colour of the indicator due to the growth of the survivors and their action on the mannitol.

TABLE III.—*Bactericidal power (Wright's method). Figures represent the strongest dilution of a 24 hours old broth culture killed by an equal volume of serum.*

Experiment I.
Injections: 500 millions April 17th, 1000 millions April 27th, subcutaneous.

	Heated vaccine.		Acetone-ext. vaccine.	
Date.	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.
Apr. 17 .	100,000	1,000,000	100,000	1,000,000
,, 18 .	1,000,000	1,000,000	1,000,000	1,000,000
,, 19 .	1,000,000	1,000,000	1,000,000	1,000,000
,, 20 .	10,000	10,000	1,000,000	100,000
,, 21 .	1,000	1,000	100,000	300,000
,, 22 .	1,000	1,000	10,000	3,000
,, 24 .	100	100	300	1,000
,, 25 .	100	100	30	100
,, 27 .	300	100	30	100
,, 28 .	300	30	30	100
,, 29 .	100	10	100	100
May 1 .	300	30	100	100
,, 3 .	100	30	10	3
,, 4 .	10	100	30	30

Experiment II.
Injections: 500 millions May 2nd, 1000 millions May 15th, both subcutaneous.

	Heated vaccine.		Acetone-ext. vaccine.	
Date	Rabbit A.	Rabbit B.	Rabbit C.	Rabbit D.
May 2	1,000,000	1,000,000	30,000	300,000
,, 4 .	100,000	1,000,000	100,000	3,000
,, 5 .	1,000	1,000,000	1,000	1,000
,, 6 .	10	100	100	1,000
,, 8 .	10	100	30	300
,, 9 .	3	30	3	300
,, 11 .	10	10	100	100
,, 13 .	10	10	10	100
,, 15 .	10	30	10	100
,, 17 .	100	30	300	100
,, 18 .	100	100	100	100

Tested by Wright's method the rabbits in the first experiment showed no marked difference whether they were injected with the acetone-extracted or the ordinary vaccine, as is seen in Table III. In the second experiment there is a slight advantage in the ordinary vaccine (Table III). Tested by the serum dilution method the same slight benefit as regards the ordinary vaccine is seen (Table IV).

Table IV.—*Bactericidal power. (Serum dilution method.) Figures represent the dilution of serum which killed all the bacilli in an equal volume of* 1 *in* 100,000 *dilution of a* 24 *hours old broth culture of* B. typhosus.

Experiment I.
Injections: 500 millions April 17th, 1000 millions April 27th, subcutaneous.

Date	Heated vacine.		Acetone-ext. vaccine.	
	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.
Apr. 17	2	2	2	2
„ 18	2	4	4	2
„ 19	4	4	4	2
„ 20	4	2	2	2
„ 22	16	2	4	2
„ 24	16	8	8	8
„ 25	16	8	8	8
„ 27	16	8	4	8
May 1	16	16	32	32
„ 3	32	32	32	64
„ 4	16	16	16	16
„ 6	Died	—	—	—
„ 8	—	32	32	32
„ 10	—	16	32	32

Experiment II.
Injections: May 2nd 500 millions, May 15th 1000 millions, both subcutaneous, May 24th 1000 millions, intravenous.

Date	Heated vaccine.		Acetone-ext. vaccine.		Average of 2 normal rabbits.
	Rabbit A.	Rabbit B.	Rabbit C.	Rabbit D.	
May 2	4	2	4	2	3
„ 3	8	8	8	4	6
„ 4	8	2	8	2	6
„ 5	8	4	8	4	3
„ 6	8	8	8	4	2
„ 7	32	16	32	8	4
„ 8	32	32	16	32	4
„ 10	32	32	16	16	2
„ 12	32	32	32	8	2
„ 13	64	32	32	8	2
„ 15	32	32	32	16	2
„ 17	16	16	16	8	2
„ 18	8	8	8	4	2
„ 20	4	2	4	2	2
„ 25	4	8	16	2	2
„ 26	8	4	8	4	2
„ 29	4	2	4	2	2
„ 31	2	2	2	2	3

Table V.—*Opsonic indices of rabbits inoculated with vaccines made from heated and acetone-extracted* B. typhosus. *Serum heated to* 60^{c} *C. for* 10 *minutes. Injections of* 500 *millions and* 1000 *millions given subcutaneously on May* 2nd *and May* 15th *respectively. The indices were obtained by using the count obtained from the unheated serum of a normal rabbit as the denominator.*

Date.	Heated vaccine.		Acetone-extracted vaccine.	
	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.
May 1	0	0	0	0
„ 2	0	0	0	0
„ 3	0	0	0	0
„ 4	0	0·06	0·05	0·03
„ 5	0	0	0·04	0
„ 6	0·10	0·15	0·12	0·20
„ 7	0·35	0·30	0·36	0·48
„ 8	0·48	0·69	0·53	0·75
„ 9	0·52	0·89	0·60	0·91
„ 10	0·56	0·95	0·61	0·89
„ 11	0·78	0·92	0·95	0·99
„ 12	0·76	0·91	0·89	1·00
„ 14	0·75	0·82	0·91	1·00
„ 16	0·63	0·85	0·80	1·10
„ 17	0·53	0·81	0·79	1·23
„ 19	0·61	0·68	0·95	0·96
„ 20	0·50	0·51	0·81	0·84

(*c*) *Opsonic power.*—There were considerable technical difficulties in the way of obtaining accurate readings when dealing with the unheated serum, but when the serum was heated to 60° C. for ten minutes the test could be more accurately made than when the usual technique was employed. From Table V it will be seen that there was not a great difference in the results following the two varieties of vaccine, but what difference there was was in favour of the one extracted with acetone.

Summary of the results obtained with B. typhosus.—The results of the agglutination tests showed that there was a slight difference in favour of the acetone-extracted vaccine. The same result was obtained with the opsonic power, whereas in the bactericidal tests there was a slight advantage in favour of the ordinary vaccine. The net result appears to be that there is no real difference in the amount of antibodies produced, whether the vaccine is prepared in the usual way, or whether acetone-extracted bacilli are employed.

Staphylococcus.

One experiment was carried out with this organism, using two rabbits inoculated with ordinary staphylococcus vaccine and two other rabbits inoculated with acetone-extracted staphylococcus. In neither case could agglutinins or bactericidal substances be detected in the serum of the inoculated animals. The opsonic power of these rabbits to staphylococcus was tested daily, and the results are shown in Table VI. It will be seen that in neither case was there a very marked alteration of the opsonic power as a result of the inoculations, and while the mean of the opsonic powers of the rabbits inoculated with the ordinary vaccine was a little higher after the first inoculation, the case was reversed after the second inoculation, when the mean of the opsonic power of the rabbits inoculated with the acetone-extracted vaccine reached a higher level.

TABLE VI.—*Opsonic indices of rabbits inoculated with vaccines made from heated and acetone-extracted Staphylococci. Injections of* 500 *millions,* 1000 *millions and* 2000 *millions given subcutaneously on June* 1*st,* 8*th and* 15*th respectively, and* 10,000 *millions intravenously on June* 29*th.*

	Heated vaccine.		Acetone extracted vaccine.	
Date	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.
June 1	1·16	1·46	1·62	1·08
„ 2	1·03	0·92	0·48	0·97
„ 3	1·05	0·80	0·96	0·67
„ 5	1·20	0·95	0·90	0·97
„ 6	1·27	1·36	1·31	1·13
„ 7	1·01	0·9	1·08	1·03
„ 8	1·11	0·69	1·07	0·83
„ 9	0·62	0·56	0·66	0·88
„ 12	0·70	0·99	1·24	1·00
„ 13	1·17	1·34	1·55	1·43
„ 15	1·54	1·17	1·20	0·90
„ 16	1·38	1·07	1·13	—
„ 19	1·80	1·04	1·05	—
„ 20	1·15	1·06	0·96	—
„ 24	1·08	0·92	1·00	—
„ 27	1·10	1·01	0·95	—
„ 29	0·83	—	0·90	—
„ 30	0·88	—	1·07	—
July 1	1·02	—	1·00	—
„ 3	1·05	—	0·70	—
„ 4	0·76	—	1·12	—
„ 7	0·94	—	1·39	—
„ 14	1·48	—	1·78	—

B. welchii.

One experiment was made with this organism. The results were, however, negative, as no agglutinins or bactericidal substances could be demonstrated, and the opsonic results were not satisfactory owing to technical difficulties encountered, which had not been overcome when the work was discontinued.

THE USE OF ACETONE-EXTRACTED BACTERIA AS AN ANTIGEN FOR COMPLEMENT FIXATION TESTS.

A large series of experiments were carried out with *B. typhosus* as the test organism in which the acetone-extracted bacilli were compared with living, formolised, and bacilli killed by heat. The serum employed in the tests was obtained from normal rabbits and also from those which had received inoculations of acetone-extracted bacilli and vaccine sterilised by heat.

The result of all these experiments was uniformly in favour of the acetone-extracted bacilli. The result of a typical experiment is given below. The technique employed was similar to that described by one of us (A. F.) for carrying out the Wassermann reaction with minimal quantities of serum.

The antigens used were (i) a suspension of living *B. typhosus*, or (ii) a suspension of the same strain of bacilli extracted with acetone and of equal opacity. Each tube was filled with:

Complement, 10 c.mm. of fresh normal rabbit's serum.

Test-serum, 20 c.mm. of normal or immune rabbit's serum, which had been heated to 56° C. for 30 minutes.

Antigen, 50 c.mm. of various dilutions of one of the above suspensions.

After mixing the tubes were incubated for one hour at 37° C., and then 10 c.mm. of sensitised sheep's corpuscles were added, and the tubes again incubated for two hours at 37°C.

TABLE VII.

Test serum employed.	Acetone-extracted bacilli.						Living bacilli.					
	Dilutions of antigen.											
	1 in:						1 in:					
	2.	4.	8.	16.	32.	Control.	2.	4.	8.	16.	32.	Control.
(1) Immune rabbit No. 1	0	0	0	0	0	C	0	0	0	P	P	C
(2) Immune rabbit No. 2	0	0	0	P	P	C	0	0	0	P	AC	C
(3) Normal rabbit	C	C	C	C	C	C	0	0	0	AC	C	C

O indicates *no* hæmolysis. P indicates *partial* hæmolysis. C indicates *complete* hæmolysis. AC indicates *almost complete* hæmolysis.

THE AGGLUTINABILITY OF *B. TYPHOSUS* AFTER EXTRACTION WITH ACETONE.

Experiments were carried out by making suspensions of equal opacity of acetone-extracted bacilli and living organisms obtained from a twenty-four hours old agar slope culture of *B. typhosus* and then adding to these a strongly agglutinating serum in various dilutions. The mixtures were then incubated at 50° C. for several hours. It was found that, with the acetone-extracted bacilli, there was a great delay in the reaction. After being incubated for one hour at 50° C. no agglutination could be detected even in a 1 in 50 dilution. After longer periods, however, agglutination took place, but never in such high dilutions of the serum as was the case when living emulsions were employed.

TABLE VIII.

Emulsion employed.	Dilutions of serum. 1 in:							Control.
	400.	800.	1600.	3200.	6400.	12,800	25,600	
Living *B. typhosus*	+	+	+	+	+	+	+	—
Acetone-extracted *B. typhosus*	+	+	+	+	+	—	—	—

THE EFFECTS OF INOCULATING RABBITS WITH ACETONE EXTRACTED *B. TYPHOSUS*, DIGESTED WITH TRYPSIN.

Only a single tentative experiment was made to ascertain the antigenic properties of bacilli which had been digested with trypsin. In this experiment one rabbit was inoculated with a dose of undigested acetone-extracted bacilli, while four others were inoculated with the same quantity of vaccine, which had been digested with 1 per cent. trypsin at 50° C. for 5, 10, 30 and 60 minutes respectively. One other rabbit was inoculated with the same quantity of vaccine which had been kept in the water-bath at 50° C. for 60 minutes. The agglutinating power of the serum of each rabbit was tested daily for six days, the technique employed being the same as in the former experiments, and two estimations were also made of the bactericidal power, one before the inoculation, the other on the fifth day after the inoculation.

The results are shown in Tables IX and X:

TABLE IX.—*Agglutination power. Injections given on July 12th. The figures given represent the highest dilution of the serum which gave standard agglutination after one hour at* 50° C. *in the case of the agglutinating power, and the highest dilution of the serum which killed an equal quantity of* 1 *in* 100,000 *dilution of a twenty-four hours old broth culture of* B. typhosus.

Date.	Type of vaccine.					
	Undigested.	Digested for 5 minutes.	Digested for 10 minutes.	Digested for 30 minutes.	Digested for 60 minutes.	In water-bath for 60 minutes.
July 12	0	0	0	0	0	0
„ 14	0	0	0	0	0	0
„ 15	20	5	5	5	5	20
„ 16	80	5	20	10	10	40
„ 17	160	5	80	10	40	40
„ 18	800	50	300	150	150	800

TABLE X.—*Bactericidal power.*

Date.	Type of vaccine.					
	Undigested.	Digested for 5 minutes.	Digested for 10 minutes.	Digested for 30 minutes.	Digested for 60 minutes.	In water-bath for 60 minutes.
July 12	2	2	2	2	2	2
„ 17	4	8	8	8	4	8

From these experiments it is obvious that the power of producing agglutinins is very considerably reduced by tryptic digestion, while as regards the power of producing bactericidal bodies there is no diminution on digesting the vaccine for one hour at 50° C. with 1 per cent. trypsin.

CONCLUSIONS.

(1) Acetone-extracted bacilli form a convenient way of storing bacilli and apparently keep their antigenic properties unchanged indefinitely.

(2) They are very easily dissolved by tryptic and other proteolytic ferments.

(3) When employed as vaccines, acetone-extracted bacilli appear to have antigenic properties fully as great as vaccines made by other methods.

(4) They also form a very suitable antigen for use in complement fixation tests.

(5) Suspensions of these extracted bacilli are agglutinated by immune sera only after a long period and by dilutions more concentrated than is the case with suspensions of living or formolised bacilli.

(6) Preliminary experiments appear to show that acetone-extracted bacilli which have been digested with trypsin when injected into animals produce a marked increase (equal to or greater than undigested bacilli) of the bactericidal power, but the increase of the agglutinating power is very much less marked.

CRITICAL REVIEW.

THE MORBID HISTOLOGY, BACTERIOLOGY AND EXPERIMENTAL PATHOLOGY OF *ENCEPHALITIS LETHARGICA.*

A. SALUSBURY MACNALTY.

Received for publication April 4th, 1921.

WHATEVER views are held as to the occurrence in the past of outbreaks of *Encephalitis lethargica*, the pathological study of the malady dates from the remarkable outbreak in the winter of 1916–17 described by von Economo.(1) The clinical symptoms of thirteen cases presenting the phenomena of stupor accompanied by disturbances of the ocular muscles were given in detail, while von Wiesner (2) made himself responsible for the pathological and experimental work carried out on five fatal cases.

Encephalitis lethargica next made its appearance in England and France. In the latter country the disease was studied by Netter (3, 4), who recorded histological findings identical with those of von Wiesner. In England, in the course of the months March to April, 1918, a certain number of cases of an obscure disease characterised usually by stupor or lethargy and ophthalmoplegia were reported, early attention being directed to the subject by Harris (5) and by Hall.(6) A likeness to the clinical description of "botulism" was observed in these cases, and the suggestion was hazarded that they were due to the consumption of food infected with the *B. botulinus* of van Ermengem. A detailed investigation of the outbreak was made under the auspices of the Local Government Board and the Medical Research Committee with the following results:

In the first place, on the strength of the bacteriological investigations of McIntosh (7), which accorded with the epidemiological inquiries, botulism was ruled out of court as responsible for the outbreak.

Secondly, in the histological researches on certain of the fatal cases carried out by McIntosh and Marinesco (8), both workers arrived independently at the conclusion that this obscure disease, as it appeared in the outbreak, was identical with the illness described by von Economo in Austria and by Netter in France, and that it is a disease anatomically and clinically distinct from analogous affections.

An epidemiological research was carried out by James (9) and a clinical study was made of the outbreak by MacNalty (10); from independent standpoints and by pursuing different lines of inquiry these investigators came to the same conclusion, regarding the disease as did Marinesco and McIntosh. Evidence supporting this view was also adduced by Kinnier Wilson (11) and Buzzard (12).

Encephalitis lethargica appears to be quite distinct from the epidemic which occurred in the late summers of 1917 and 1918 in New South Wales. Cleland and Campbell (13) report that microscopical examination of the nervous system in the cases showed a general acute encephalo-myelitis. In the autumn of 1918 cases of *Encephalitis lethargica* were recorded in the United States, and Bassoe (14) found the usual pathological picture of the disease.

The more recent work upon the pathology of *Encephalitis lethargica* has been carried out by McIntosh and Turnbull, Greenfield (15) and Da Fano and Ingleby in this country, in France by Levaditi and Harvier, and in the United States by Loewe, Hirshfeld and Strauss.

CERTAIN ANATOMICAL AND PHYSIOLOGICAL CONSIDERATIONS BEARING UPON THE PATHOLOGY OF THE DISEASE.

MacNalty (10) has offered the following observations relating to the pathology of *Encephalitis lethargica.*

The preponderating lesion in the disease is usually in the mesencephalon, in or about the nucleus of the third nerve. The nucleus of the third nerve and certain of its emerging fibres bear intimate relation to the afferent fibres streaming towards the thalamic region, neopallium, etc. Hence any illness affecting this anatomical localisation of afferent paths is associated with stupor, for it involves a blockage of the afferent stimuli. The association of stupor and ophthalmoplegia is thus explained. If, however, the chief stress of the disease falls outside the third nucleus and its emerging fibres, or if the disease terminates too quickly to allow of involvement of the third nerve being apparent, in either case there will be interference with the afferent paths conveying environmental stimuli, and the phenomenon of stupor or drowsiness without localising signs will obtain. This pathological distribution will account for two groups of the disease recognised clinically, namely, cases with general symptoms and without localising signs, and cases with localising signs in the central nervous system. In addition, the explanation is adequate for the group of cases with mild or transient manifestations. The anatomical localisation is common for all three groups, but in the one last mentioned the intensity of the interference is diminished.

The anatomical relationship of the paths of the rubro-spinal and the pyramidal tracts to the nuclei of the third nerve explains the tremors (affection of the rubro-spinal fibres) and the presence of Babinski's sign seen in certain cases of *Encephalitis lethargica.*

In the group of cases of the disease with nervous localising signs clinical evidence shows that certain types occur displaying as a rule stupor but with no ophthalmoplegia. Paralytic phenomena are exhibited, one type being associated with local lesions of cranial nerves other than the third, the seventh nerve being notably affected, and another type with spinal manifestations.

Pathological evidence furnishes similar proofs to the effect that although lesions in or about the nuclei of the third nerve preponderate, yet frequently they are not limited to this region, but may involve or be primary in the pons, bulb, the cerebellum (Marinesco), the cerebral cortex, as described by Buzzard and Greenfield ([15]) and by Watson ([16]), and, rarely, the spinal cord.

A series of polyneuritic cases was clinically described in the outbreak of 1918. In the absence of pathological information it is uncertain whether such cases are anatomically best explained as of mesencephalic origin with a secondary toxic effect upon the peripheral nerves, or whether a primary or concomitant spinal lesion was present.

Although, then, the anatomical situation of the lesion of *Encephalitis lethargica* is usually mesencephalic and in or near the nucleus of the third nerve, in an individual case the preponderating lesion may be elsewhere in the central nervous system, so that to complete the histological study it is desirable to make a complete investigation of the brain and spinal cord.

POST-MORTEM FINDINGS.

The *post-mortem* examinations carried out at the Pathological Institute of the Vienna University revealed no noticeable changes in the internal organs. McIntosh ([7]), who analysed a number of *post-mortem* examination reports in the English outbreak of 1918, found no macroscopic changes present that would be sufficient to account for death. He observed that many of the cases showed some congestion of the brain, increase of cerebro-spinal fluid, with a dilatation of the vessels of the pons and the thalamic region; no actual hæmorrhages into the brain-substance were seen but two superficial hæmorrhages were recorded.

Buzzard ([12]) would recognise a group of cases in which the encephalitis has its chief incidence on the cerebral hemispheres and in which hæmorrhage is a conspicuous feature.

MORBID HISTOLOGY.

The morbid histology of *Encephalitis lethargica* has been carefully studied by von Wiesner ([2]), Netter ([3, 4]), McIntosh and Turnbull ([7]) and Marinesco.([8]) Their findings harmonise with one another, and as a result the main histological picture of the disease can be regarded as clearly delineated.

The lesion is definitely inflammatory, and the changes consist of a cellular infiltration of the perivascular lymphatic sheaths and of certain areas of grey matter (McIntosh).

There is no marked change in the meninges; insignificant increases of the cellular elements of the pia arachnoid, particularly in the neighbourhood of the blood-vessels, have been observed. In three cases of *Encephalitis lethargica* examined by Bassoe and Hassin ([17]) there was a mild leptomeningitis, the vessels of the pia being congested with perivascular infiltration with plasma-cells and leucocytes.

In the brain substance, as already mentioned, the changes are most conspicuous in the upper part of the pons and in the basal nuclei. In the spinal cord the lesions are very slight or entirely absent. The lesion in the grey matter is focal in character; it consists of what von Wiesner terms "colossal" small-celled infiltration of the perivascular lymphatic sheaths and of the parenchyma. Marinesco points out that the endothelium and fibroblasts may also have taken part in the inflammatory process. The parenchyma may be œdematous; the extent of infiltration here varies considerably, but it is usually proportional to the perivascular lesion. According to McIntosh, the cellular exudate is composed chiefly of large and small mononuclear lymphocytes, along with a certain number of the polyblasts of Maximow and a few plasma-cells. In most of the cases the small or medium-sized veins were most affected. McIntosh states that polynuclear leucocytes of any variety are noticeably absent; von Wiesner, in his cases, detected "nests" or small groupings of polynuclear leucocytes in isolated spots of the parenchyma. The extra-adventitial infiltration is usually diffuse, but bearing a relation to the perivascular infiltration is largely due to emigrated cells in McIntosh's opinion. At the same time proliferation of the neuroglial cells undoubtedly occurs. In certain areas, particularly in the basal nuclei and upper parts of the pons, McIntosh found the infiltration might be so dense as to form actual foci; such foci resemble those seen in the grey matter of the cord in poliomyelitis, but one striking difference between the two forms of disease is that in the cases examined in the 1918 epidemic the ganglion cells were still present in considerable numbers. Marinesco describes foci of interstitial inflammation consisting of neuroglia cells of various kinds, including large cells with voluminous eccentric nuclei and many fibrillar prolongations, lymphocytes and polynuclears. Such foci have been noticed by von Wiesner and Marinesco as sometimes occurring in the roots of the cranial nerves, *e. g.* hypoglossal, pneumogastric. Marinesco considers that the interstitial changes may be independent occasionally of vascular infiltrations, and Bassoe and Hassin have come to a similar conclusion. Both von Wiesner and McIntosh note that there is no special involvement of the ganglion cells, which, as a rule, are well defined with the Nissl granules present. In the areas of nervous tissue most affected some of the ganglion cells may be globular in shape with the chromatin diffuse and some mononuclear cells in close proximity, but neuronophagia is extremely rare and when seen is never marked.

Marinesco has described degenerative changes in the cells of Purkinjë in a case of *Encephalitis lethargica* in which the cerebellum was involved.

In a preliminary communication Da Fano and Ingleby ([18]) confirmed the histological observations of previous workers, but laid stress on alterations in the nerve-cells. Their cases of *Encephalitis lethargica* showed changes ranging from slight chromatolysis to complete disappearance of Nissl granules, atrophy of the protoplasm and shrinkage, atrophy and final disappearance of the nucleus. They also directed attention to the presence of a granular pigment-like material occurring within the nerve-cells in places where brown or black pigment is not generally found. In a further communication Da Fano ([19]) reports that within and without the nerve-cells minute forms have been observed, to all appearance consisting of a central generally basophil particle and of a delicate little stainable body, irregularly round or oval in shape. These he terms "minute bodies." They are generally discrete and provided with one granule, but dumbbell-shaped forms occur, as well as others with two central particles arranged in pairs. A relation, as yet not defined, appears to exist between these forms and the granular pigment-like material. Similar "minute bodies" have been traced within and without the cells infiltrating a salivary gland from an acute case of *Encephalitis lethargica.*

After discussing the supposition that such findings might be associated with degenerative changes caused by the malady, Da Fano tentatively puts forward the suggestion that they may be due to the presence in the tissues of a living agent, the cause of the disease, on the analogue of the Negri bodies in rabies.

In this connection one may note that Levaditi, Harvier and Nicolau (35) have found in rabbits which have died of *Encephalitis lethargica*, produced by cerebral inoculation, corpuscles in the cerebral tissues which they describe as similar to Negri bodies.

According to von Wiesner and to McIntosh, hæmorrhages are usually absent in the sections examined. Netter in his series of cases, however, found that the grey and white matter might be the seat of numerous hæmorrhages ("piqueté hæmorrhagique"). In two brains examined by Marinesco hæmorrhagic foci were numerous; nevertheless the vessel-wall did not appear to be necrosed, but sometimes a solution of continuity of the vessel-wall could be seen.

Netter states that the salivary glands in cases of *Encephalitis lethargica* may be affected.(20) Netter and Durand (20) have examined systematically the parotid and submaxillary glands from fatal cases of the disease, and find that round-celled infiltrations are very numerous in histological sections of the glands.

Such is the outline of the characteristic morbid histology of *Encephalitis lethargica.* The stages in the development of the lesion require yet to be worked out.

Perhaps the tentative suggestion may be made that the inflammatory lesion is somewhat slow of manifestation. In Lereboullet and Hutinel's case (21), which proved fatal after a week's illness, the histological findings were almost completely negative, although the clinical symptoms were typical; in a case of *Encephalitis lethargica* without localising signs which proved fatal within six days, reported by MacNalty (10), Graves noted only a few areas of round-celled infiltration in the basal ganglia with marked distension of the small cerebral vessels suggesting an early stage of the disease; and in four of MacNalty's acute cases from the Derby outbreak, proved experimentally to be one of *Encephalitis lethargica*, McIntosh and Turnbull and Da Fano and Ingleby reported negative findings as regards microscopical examination of the cerebral tissue. All four cases proved fatal within a few days.(22)

BACTERIOLOGICAL FINDINGS.

Examination of material from cases of *Encephalitis lethargica* during life has been undertaken by von Wiesner (2) and by McIntosh.(7) The examination of the cerebro-spinal fluid showed no specific changes. In some of the cases it was under a slightly increased pressure, and in others there was a slight increase in the number of lymphocytes (McIntosh). No organisms could be found in any of the specimens, and the Wassermann reaction, both in the cerebro-spinal fluid and the serum of the patients, was negative in every case. The blood taken by veni-puncture was in each instance sterile. Cultures of the naso-pharynx failed to demonstrate the constant presence of any particular micro-organism. From all fatal cases examined by him von Wiesner recovered a Gram-positive organism which he termed a diplo-streptococcus, but the work of subsequent investigators has not confirmed his findings, the prevailing opinion being that he was dealing with a contamination.

Working at the Mount Sinai Hospital, New York, Strauss, Hirshfeld and Loewe ([23]), and later Loewe, Hirshfeld and Strauss ([24]), have obtained a filtrable virus from the naso-pharyngeal membrane of fatal cases of *Encephalitis lethargica* which they regard as the specific agent of the disease. The virus has been passed in series through monkeys and rabbits. It can be preserved for many months in 50 per cent. glycerol. In a later communication Loewe and Strauss ([25]) claim that by means of the ascitic-tissue culture methods perfected by Noguchi they have been able to cultivate a minute filtrable organism from cases of *Encephalitis lethargica;* this organism is present in the brain, naso-pharyngeal mucous membrane, naso-pharyngeal washings, spinal fluid and blood. A successful growth is usually manifested on the fifth to seventh day by clouding of the medium. Transfer to solid media can be obtained only with the later generations of the organism. Studics of the fluid cultures under dark field illumination reveal the organisms as minute globular refractile forms, occurring singly, doubly or in chains and clumps, the latter form predominating especially in the older cultures. These bodies show active Brownian movement, but no true motility. The organism has an average diameter of 0·25 mikrons. The curious statement is made that the organism may be Gram-positive or Gram-negative; it stains by Giemsa and by methylene-blue. Thalimer ([26]) claims to have confirmed the work of Loewe and Strauss, but otherwise it is at variance with the results of English and French bacteriologists. In general it may be said that the remarkable similarity between the experiments of these authors and of those who recently claimed to have discovered a culturable filter-passing virus as the cause of influenza and other diseases enforces an attitude of reserve. Kling and Liljenquist ([36]) in Sweden claim to have found the virus of *Encephalitis lethargica* in the nasopharyngeal secretions and in the intestinal contents, but the evidence on which they base their assertion is not yet available.

McIntosh ([27, 28]) in 1919 showed by his experimental results that the disease was caused by a living virus, in all probability a filtrable virus.

Levaditi and Harvier ([29]), from the cerebral and spinal nervous tissue of rabbits inoculated with *Encephalitis lethargica* from human cases of the disease occurring in the Paris outbreak of 1919–20, have isolated a filtrable virus which can pass wholly or partially through Chamberland filters Nos. 1 and 3. This causal virus can be preserved for some length of time in glycerine, is destroyed at 56° C., is killed by prolonged contact with phenol and preserves its virulence for at least forty-eight hours after death. In spite of numerous attempts they have not been able to cultivate the organism on Noguchi's medium, but they have succeeded in preserving its virulence at 37° C. in association with cells of the testicle of the rabbit cultivated *in vitro*.

Attenuated varieties of the organism exist.

In a communication of recent date, Levaditi, Harvier and Nicolau (35) give some further particulars relative to their experiments with the virus:

(1) They find that the serum of human subjects convalescent from *Encephalitis lethargica*, although it only rarely and partially neutralises the virus *in vitro*, yet may be shown to possess specific qualities. Two volumes of serum (in one instance convalescent serum and in the other normal human serum) are mixed with one volume of virus and kept in contact for five hours at 37° C. The rabbit inoculated on the cornea with the mixture of virus and convalescent serum does not exhibit keratitis and survives, while the rabbit inoculated with the mixture of virus and normal serum develops keratitis and dies of encephalitis. This may lead to the application of a "kerato-diagnostic" method. The serum of a sheep which has been given subcutaneous injections of virulent emulsions of the brains of rabbits dead from *Encephalitis lethargica* is able to neutralise the virus *in vitro*. It gives a positive Bordet-Gengou reaction with the infected brain which is much more intense than with the normal brain. Levaditi, Harvier and Nicolau anticipate that this serum will enable them to differentiate the virus of *Encephalitis lethargica* from other similar viruses such as rabies and poliomyelitis.

(2) They find that anæsthetics, particularly chloroform, ether and chloral, have a manifest and appreciable effect in increasing the pathogenicity of the virus inoculated intra-cerebrally.

(3) They have established the following new properties of the virus: (*a*) Prolonged and intense centrifugalisation of a cerebral emulsion of the virus does not deprive the supernatant liquid of its pathogenic power.

(*b*) The virus emulsion is still active for intra-cerebral inoculation after dilution up to 1/1000 in physiological saline.

(*c*) The virus dried *in vacuo* is still pathogenic after preservation for forty days at room temperature. Mixed with milk, it still remains virulent after forty days' preservation under the same conditions of temperature.

(*d*) A fragment of virulent brain placed in glycerine does not contaminate the glycerine medium or pieces of normal brain or normal kidney with which it is placed in contact. This experiment joined to others made with solid gelatine covered with virus emulsion proves that the virus is not diffusible.

EXPERIMENTAL INVESTIGATIONS.

In 1917 von Wiesner (2), with material obtained from von Economo's cases, produced a hæmorrhagic encephalitis in a monkey. He recovered his diplo-streptococcus from the brain of the lethargic monkey. Considerable doubt remains as to the conclusions to be drawn from this early experiment.

When studying the outbreak of *Encephalitis lethargica* in 1918, McIntosh (7) inoculated, intracerebrally, material from eight of the fatal cases into *Macacus rhesus* monkeys, and clearly showed the relative insusceptibility of this monkey to the virus as compared with that of poliomyelitis. In no case did any definite paralysis develop. Then in 1919, Strauss, Hirshfeld and Loewe produced an acute encephalitis in monkeys by intra-cerebral inoculations of material derived from cases of *Encephalitis lethargica*. The type of lesion observed and the short incubation period suggest that in all probability these workers were dealing with another infection. Later they successfully transmitted the disease to rabbits and to monkeys.

In 1919 McIntosh succeeded in transmitting *Encephalitis lethargica* to a *Patas* monkey. In August of that year an institutional epidemic of *Encephalitis lethargica* occurred in a home for girls at Derby.(22) Out of twenty-one inmates of the home there were twelve cases with five deaths. A *Patas* monkey, inoculated by McIntosh with material from one of the fatal cases, presented lethargic symptoms and tremors and died. On examination, Turnbull (27) found that the monkey's brain showed lesions similar to those found in human cases of *Encephalitis lethargica*. The monkey was inoculated intra-cerebrally and intra-peritoneally. Pieces of the cervical cord, pons and basal nuclei, each about the size of a large pea, were placed in a conical glass vessel, and, after the excess of glycerine had been washed off with sterile saline, were pounded up by means of a glass rod. The pounded material was then emulsified in 20 c.c. of sterile saline. About 10 c.c. of this emulsion was diluted with saline until it could be drawn through a Berkefeld filter with the aid of an exhaust pump. The *Patas* monkey was injected with the filtered emulsion. Subsequently McIntosh and Turnbull (28) have reported the successful transmission in series of experimental *Encephalitis lethargica* to monkeys and rabbits, which has completed the evidence necessary to show that the disease is caused by a living virus. Additional proof was provided by the spontaneous infection with *Encephalitis lethargica* of a *Macacus cynomolgus* monkey kept as a control along with an inoculated monkey.

As McIntosh and Turnbull pointed out, this was the first indisputable occasion upon which experimental transmission of *Encephalitis lethargica* to an animal was successful, and the experiments indicate therefore a conspicuous advance in our knowledge of the disease.

Commencing experimental researches in December, 1919, Levaditi and Harvier (29) for some time essayed without success to transmit *Encephalitis lethargica* to monkeys and rabbits. In February, 1920, they succeeded by using an emulsion of cerebral and spinal tissue, obtained from a fatal case of the disease, in transmitting the malady to a rabbit. As already mentioned, by this experiment they obtained a specific filtrable virus which they regard as the causal agent of the disease, thus confirming the experimental results of McIntosh.

Levaditi and Harvier state that this virus is pathogenic for the rabbit and guinea-pig but it is either slightly virulent or non-virulent for the monkey. The lesion which this virus produces in the affected animals and also the symptoms of the experimental disease are practically identical with those in the human subject. They find that *Encephalitis lethargica* may be transmitted to the rabbit intra-cranially, through the orbit and by inoculation of the virus into a peripheral nerve such as the sciatic. The virus is harmless when injected subcutaneously, intravenously, into the peritoneum, into the trachea, into the alimentary canal or into the parenchyma of the salivary gland; if injected into the testicle, however, it produces *Encephalitis* *lethargica* in the experimental animal, and retains its virulence in the testicle for at least seventeen days. Levaditi and Harvier further find that the intact nasal mucous membrane offers a barrier to the penetration of the organism, and that this protection is only overcome when the nasal mucous membrane is injured and inflamed. The organism follows then, probably, the ascending path of the olfactory nerves in order to reach the brain. The virus exists in the cerebrum and in the spinal cord of the subjects of *Encephalitis lethargica* but is absent from the blood, cerebro-spinal fluid and the organs of the body, including the salivary gland.

Netter (20), from his histological observations, considered that the virus was probably present in the salivary glands, and that the saliva was the chief source of infection. Levaditi and Harvier consider that the chief route of infection is by way of the naso-pharynx. In addition to the above important findings they have also conducted certain immunological experiments. They find that there is no spontaneous cure in the experimentally-produced malady, and consequently no succeeding refractory stage; nevertheless, immunity can be artificially established by living or dead vaccines; it is not an absolute immunity, but is manifest to a sufficient extent to permit of further researches into the subject. The serum of vaccinated animals as a rule is neither endowed with microbicidal properties *in vitro* nor can it act prophylactically. The same usually holds true of the sera of persons convalescent from *Encephalitis lethargica*, according to Levaditi and Harvier's studies. This lack of protective value in the sera of these patients has been confirmed by Amoss. (30) In his tests he used the blood-serum of four cases, one serum being taken from a patient convalescent in the fifth week of the disease, the second in the fourth month, the third in the fifth month, and the fourth from a patient fifteen months after the attack. He compared his results with controls and with the effect of sera from poliomyelitis patients which protected against inoculation with the virus of poliomyelitis.

The following table summarises the important pathological points of differentiation between *Encephalitis lethargica* and Acute Poliomyelitis (Heine-Medin Disease).

Differentiation between Encephalitis lethargica *and Acute Poliomyelitis.*

	Acute poliomyelitis.	Encephalitis lethargica.
Histology. Distribution of the lesions.	They are most extensive in the spinal cord (affecting the anterior cornua), and become progressively less so upwards to the brain. The pia arachnoid is frequently involved.	They are most extensive in the basal nuclei of the brain; nearly equally so in the upper part of the pons; much less extensive in the medulla; absent or very slight in the spinal cord. The pia arachnoid is only slightly involved.
Character of the infiltration.	Plasma-cells less frequent; polynuclear leucocytes more frequent. Hæmorrhages common and obvious, making the grey matter of the cord pink.	Plasma-cells numerous; polynuclear leucocytes rare. Hæmorrhages not common and microscopic.

	Acute poliomyelitis.	Encephalitis lethargica.
Changes in the nerve-cells.	Degenerative nerve-cell lesions, pronounced (neuronolysis). Motor cells surrounded and infiltrated with partially phagocytic round-cells and finally destroyed completely, leaving in their place a heap of round-cells (neuronophagia).	Perivascular infiltration is the dominant lesion. Degenerative lesions are less pronounced. As a rule the ganglion-cells are not much affected; they remain present in considerable numbers, are usually well-defined and with the Nissl granules present; Neuronophagia is very rare and when present is not marked.
Bacteriology. Virus.	A filtrable virus readily passing through Chamberland, Berkefeld, Reichel or Pukall filters (Flexner, Lewis, Leiner and Wiesner, Levaditi and Landsteiner). Resistant to freezing and to glycerine. Dried *in vacuo* (away from light) at room temperature it is potent for four weeks (Römer and Joseph). Heating for 30 mins. at 45°–55° C. destroys it. Appears to be desdestroyed by potassium permanganate, by hydrogen peroxide, by formaldehyde, by 1 per cent. thymol and by three days' contact with 0·5 per cent. phenol.	A filtrable virus passing wholly or partially through Chamberland filters (McIntosh, Levaditi and Harvier) can be preserved for some length of time in glycerine. Dried *in vacuo* at room temperature it is potent for forty days (Levaditi, Harvier and Nicolau). Destroyed at 56° C.; killed by prolonged contact with phenol. Preserves its virulence for at least forty-eight hours after death. Virulence preserved at 37°.C. in association with cells of the testicle of the rabbit cultivated *in vitro* (Levaditi and Harvier). It is not diffusible.
Localisation of virus.	In brain, spinal cord, cerebro-spinal fluid, mesenteric glands, tonsils, in mucous secretions of naso-pharynx, trachea and intestines. Also in dust of rooms.	Exists in brain and spinal cord. Absent from blood, cerebro-spinal fluid and organs of the body (McIntosh, Levaditi and Harvier).
Transmission to animals.	Virus readily transmissible to monkeys. Not easily transmissible to rabbits.	Virus transmissible with difficulty and only from acute cases of the disease. Pathogenic for rabbit and guinea-pig; slightly virulent or non-virulent for the monkey (Levaditi and Harvier), but virulent for certain species of monkeys (McIntosh).
Immunity. Active immunity.	Early appearance of immunity after attack of poliomyelitis. Animals immunised by gradually increasing doses of fully virulent cord (Flexner and Lewis). Monkeys actively immunised by a mixture of virus and immune serum (Landsteiner and Levaditi and Römer and Joseph).	No spontaneous cure in the experimentally produced malady (Levaditi and Harvier).
Passive immunity.	Serum of animal immunised against poliomyelitis possesses capacity of inactivating the virus *in vitro* (Levaditi and Landsteiner, Römer and Joseph, Flexner and Lewis). Immune serum protects animals against inoculation with virus, serum of convalescents protects against poliomyelitis.	Serum of animals vaccinated with the virus is, as a rule, neither microbicidal *in vitro*, nor can it act prophylactically. This is true also usually of sera of convalescent patients (Levaditi and Harvier).
Cross-immunity experiments (Amoss).	Serum of subjects of poliomyelitis does not protect against *Encephalitis lethargica.*	Serum of subjects of *Encephalitis lethargica* does not protect against poliomyelitis.

CONCLUSION.

The claims of *Encephalitis lethargica* to be regarded as an independent disease with a lesion hitherto undescribed by pathologists appear to be sufficiently well established. To this statement there is perhaps one exception. It is possible that *paralysis agitans*, or at all events certain cases of this disease, are subsequent or chronic manifestations of an unrecognised attack of *Encephalitis lethargica.* The site of election of the lesion is to some degree similar in the two diseases; the clinical symptoms of *Encephalitis lethargica*, for example the mask-like face, resemble Parkinson's disease; more convincing still, symptomatic *paralysis agitans* has been described by S. A. Kinnier Wilson ([31]), O. Crouzon ([33]) and others as following on *Encephalitis lethargica*, although Pierre Marie and Mlle. G. Lévy ([32]) consider chronic *paralysis agitans* should not be identified with such symptomatic cases.

The histological findings in *Encephalitis lethargica* are indicative of a characteristic inflammatory lesion; it is distinct, for example, from the polioencephalitis of Wernicke, which is of toxic origin. The bacteriological and experimental results indicate that the causal organism is a filtrable virus present in the tissues of the central nervous system which can be experimentally transmitted in series to monkeys and rabbits.

The pathology of *Encephalitis lethargica* is distinct from that of poliomyelitis and of the encephalitis of influenza. In the latter malady the anatomical features are invariably those of an acute hæmorrhagic encephalitis. The lesions, as Mott ([34]) and Marinesco ([8]) point out, present a striking similarity to those of the encephalitis of African sleeping-sickness due to trypanosome infection. Changes of an inflammatory nature in the grey matter of the brain are produced, and thus *Encephalitis lethargica* is comparable to the infections of syphilis, sleeping-sickness, rabies and poliomyelitis. Although comparable to these maladies in its pathological *modus operandi*, the weight of evidence has demonstrated the independent character of the disease.

REFERENCES.

1. von Economo, C.—(*a*) "Encephalitis Lethargica," *Wien. klin. Wchnschr.*, 1917, **30**, 581. (*b*) "Neue Beiträge zur Encephalitis Lethargica," *Neurol. Centralbl.*, 1917, **36**, 866. (*c*) 'Die Encephalitis Lethargica,' Leipzig und Wien, 1918 (Franz Deuticke).
2. von Wiesner, R. R.—"Die Ætiologie der Encephalitis Lethargica," *Wien. klin. Wchnschr.*, 1917, **30**, 933. See also under v. Economo.
3. Netter. A.—"L'encéphalite léthargique," *Paris méd.*, 1918, **8**, 81.
4. *Idem.*, *Bull. acad. de méd.*, 1918, **79**, 337.
5. Harris, W.—"Acute Infectious Ophthalmoplegia or Botulism," *Lancet*, 1918, **1**, 568.
6. Hall, A. J.—"Note on an Epidemic of Toxic Ophthalmoplegia," *ibid.*, 1918, **1**, 568.
7. McIntosh, J.—"Bacteriological and Experimental Investigations on Material derived from Cases of Encephalitis Lethargica," *Rep. Loc. Govern. Board on Publ. Health and Med. Subjects* (New Series), No. 121, Lond. 1918 (H.M. Stationery Office), p. 57.
8. Marinesco, G.—"Contribution to the Study of the Pathological Histology of Encephalitis Lethargica," *ibid.*, p. 47.
9. James, S. P.—"The Nature of the Disease as shown by Epidemiological Inquiry," *ibid.*, p. 39.

10. MacNalty, A. S.—"A Clinical Account of the Disease and the Opinion as to its Nature which seems justifiable from Clinical Inquiry: Report of an Inquiry into an Obscure Disease, *Encephalitis Lethargica*," *ibid.*, p. 12.
11. Wilson, S. A. K.—"Epidemic Encephalitis," *Lancet*, 1918, **2**, 7.
12. Buzzard, E. F.—"Encephalitis Lethargica," *Proc. R. Soc. Med.*, 1919, **12**, 56 (Section of Neurology), and *Lancet*, 1918, **2**, 835.
13. Cleland, J. B., and Campbell, A. W.—"Acute Encephalo-myelitis: A Clinical and Experimental Investigation of an Australian Epidemic," *Brit. Med. J.*, 1919, **1**, 663.
14. Bassoe, P.—"Epidemic Encephalitis (Nona)," *J. Amer. Med. Assoc.*, 1919, **72**, 971
15. Buzzard, E. F., and Greenfield, J. G.—"Lethargic Encephalitis: Its Sequelæ and Morbid Anatomy," *Brain*, 1919, **42**, 305.
16. Watson, G. A.—"A Case of Encephalitis Lethargica involving chiefly the Cerebral Cortex," *J. Neurol. and Psychopathol.*, 1920, **1**, 34.
17. Bassoe, P., and Hassin, G. B.—"A Contribution to the Histopathology of Epidemic ('Lethargic') Encephalitis," *Arch. Neurol. and Psychiat.*, 1919, **2**, 24.
18. Da Fano, C., and Ingleby, H.—"Demonstration of Preparations from Cases of Encephalitis Lethargica," *Proc. R. Soc. Med.*, 1919, **12**, 42 (Section of Pathology).
19. Da Fano, C.—"A Preliminary Note on the Histo-Pathology of Epidemic (Lethargic) Encephalitis," *Brit. Med. J.*, 1921, **1**, 153.
20. Netter, A.—"Altérations des glandes salivaires dans l'encéphalite léthargique," *Soc. med. d. hôp. et Acad. de Méd.*, 1920, Avril-Juillet, p. 4.
21. Lereboullet and Hutinel.—"Un nouveau cas de l'encéphalite léthargique," *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1919, 3e sér., **43**, 302.
22. MacNalty, A. S.—"Report on Outbreak of Encephalitis Lethargica in a Girl's Home," Appendix VII, *Ann. Rep. Chief Med. Officer Ministry of Health*, 1919–20, London, 1920 (H.M. Stationery Office), p. 357.
23. Strauss, Hirshfeld and Loewe.—"Studies in Epidemic Encephalitis (Encephalitis Lethargica)," *New York Med. J.*, 1919, **109**, 772.
24. Loewe, Hirshfeld and Strauss.—"Studies in Epidemic Encephalitis (Encephalitis Lethargica)," *J. Infect. Dis.*, 1919, **25**, 378.
25. Loewe and Strauss.—"Studies in Epidemic (Lethargic) Encephalitis," *ibid.*, 1920, **27**, 250.
26. Thalimer, W.—"Epidemic (Lethargic) Encephalitis, Cultural and Experimental Studies," *Archiv. Neurol. and Psychiat.*, 1921, **5**, 113.
27. McIntosh, J., and Turnbull, H. M.—"The Experimental Transmission of *Encephalitis Lethargica* to a Monkey," *Brit. J. Exper. Pathol.*, 1920, **1**, 89.
28. *Idem.*—"Transmission of Experimental *Encephalitis Lethargica* in Series in Monkeys and Rabbits, with Notes on a Spontaneous Infection in a Monkey," *ibid.*, 1920, **1**, 257.
29. Levaditi, C., and Harvier, P.—(*a*) "Recherches expérimentales sur l'encéphalite léthargique," *Compt. rend. Soc. de biol.*, 1920, **83**, 674; (*b*) "Étude expérimentale de l'encéphalite dite léthargique," *Ann. de l'Inst. Pasteur*, 1920, **34**, 911.
30. Amoss, H. L.—"Immunological Distinctions of Encephalitis and Poliomyelitis," *J. Exper. Med.*, 1921, **33**, 187.
31. Wilson, S. A. K.—"Cases of Symptomatic Paralysis Agitans following Encephalitis Lethargica," *Proc. R. Soc. Med.*, 1920, **13** (Clin. Sect.), 65.
32. Marie, P., and Lévy, G.—"Le syndrôme Parkinsonien dans l'encéphalite," *Bull. Acad. de Med.*, 1920, 3e ser., **83**, 539.
33. Crouzon, O.—"Encéphalite aiguë parkinsonienne," *Rev. de méd.*, 1920, No. 6, 359.
34. Mott, F. W.—"Discussion on Epidemic Encephalitis," *Proc. R. Soc. Med.*, 1918, **12**, 1 (Section of Medicine).
35. Levaditi, C., Harvier, P., and Nicolau, S.—"Recherches expérimentales sur le virus de l'encéphalite épidémique," *Compt. rend. Soc de biol.*, 1921, **84**, 524.
36. Kling, C., and Liljenquist, F.—"Epidémiologie de l'encéphalite léthargique," *ibid.*, p. 521.

TECHNIQUE.

AN IMPROVED FORM OF McINTOSH AND FILDES' ANAËROBIC JAR.

PAUL FILDES AND JAMES McINTOSH.

From the Bacteriological Laboratory, London Hospital, and the Bland-Sutton Institute of Pathology, Middlesex Hospital, London.

Received for publication March 31st, 1921.

THE present apparatus is an improved form of McIntosh and Fildes' anaërobic jar (1, 2, 3). It is more convenient in working and permits the action of the palladium-asbestos to be continued indefinitely to remove final traces of oxygen which have diffused out of the media.

The general arrangement will be seen in Fig. 1, and the details of the electric heater, diagrammatically, in Fig. 2.

The jar is used in the following way:

(1) Clean the opposing surfaces of lid and jar carefully with xylol. Smear on each a little tallow. This should be the best and hardest obtainable, and should be melted to allow grit to separate and then the supernatant stored in a small Petri dish. Also grease the tips and threads of the needle-valves.

(2) Place the culture-tubes inside with some lint at the bottom to prevent breakage.

(3) Prepare three stock bottles:

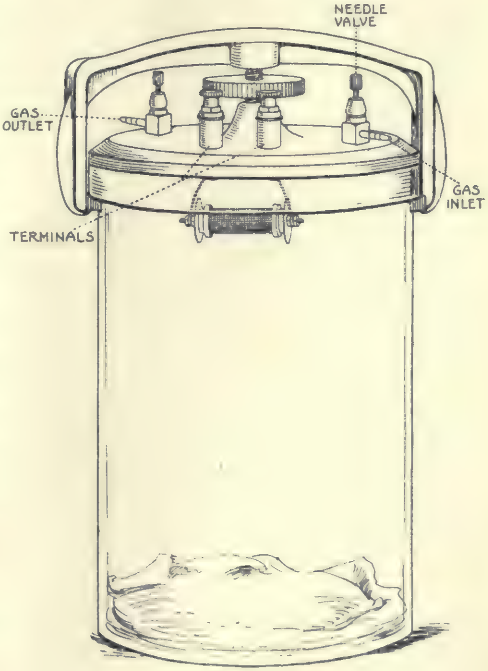


FIG. 1.

(*a*) N/10 NaOH 6 c.c., water to 100 c.c.; (*b*) $\frac{1}{2}$ per cent. watery methylene blue 3 c.c., water to 100 c.c.; (*c*) glucose 6 grm., water to 100 c.c., and a small crystal of thymol. On each occasion of closing the jar, mix equal volumes of *a*, *b* and *c* in a test-tube, boil thoroughly till reduced, and without delay place this tube in the jar as an indicator.

(4) Immediately "screw" on the lid and blow through hydrogen from a cylinder for half a minute.

(5) Shut off both valves firmly and attach the terminals to the electric supply through resistances as follows: 240 volts, three 16-c.p. carbon lamps in parallel; 200 volts, two 16- and one 8-c.p.; 100–110 volts, one 16- and one 8-c.p. lamps in parallel.

(6) In a short time dew will appear on the walls of the jar. After ten minutes there will be a negative pressure inside and more hydrogen may be added. The hydrogen cylinder should be provided with a reducing valve to deliver at the lowest possible pressure, and a mercury gauge to show the actual pressure in the delivery pipe when connected with the jar to facilitate control. The current should be continued for half an hour, and during this time and subsequently the methylene-blue tube should remain colourless except for a slight tinge at the top which slowly disappears.

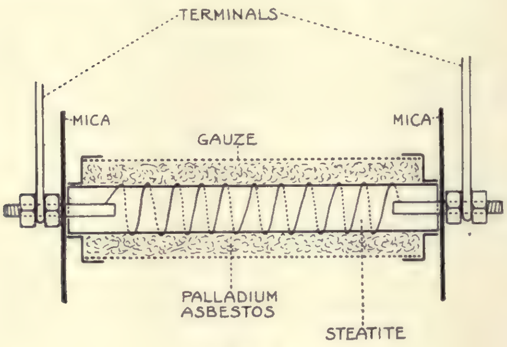


Fig. 2.

For satisfactory working the jar must be absolutely tight. If a few drops of ether are placed inside and the lid is then put on, no bubbles should escape if the jar is submerged in warm water.

If greater degrees of anaërobiosis are required the heater can be kept active by running the current continuously or at intervals after the jar has been placed in the incubator.

The apparatus is supplied by Messrs. J. J. Griffin & Sons, Ltd., Kemble Street, Kingsway, London, W.C. 2.

REFERENCES.

1. McIntosh, J., and Fildes, P.—"A New Apparatus for the Isolation and Cultivation of Anaërobic Micro-organisms," *Lancet*, 1916, **1**, 768.
2. *Ibid.*—"Nouvelle méthode d'isolement et de culture pour les microbes anaérobies," *Compt. rend. Soc. de biol.*, 1916, **79**, 293.
3. Fildes, P.—"Methods for Cultivating the Anaërobic Bacteria," *Med. Res. Comm. Spec. Rep. Series*, No. 12, London, 1917 (H.M. Stationery Office).

A NEW IMMERSION FLUID.

VILH. JENSEN.

Institute for General Pathology, Copenhagen.

Received for publication January 22nd, 1921.

In the *Journal of Pathology and Bacteriology*, 1909, **13**, p. 28, C. Rowntree has recommended liquid paraffin instead of the ordinary cedar oil for immersion lenses. This, however, cannot be done without considerable detriment to the microscopic image since the refractive index of liquid paraffin is only 1·471, while that of the specially prepared oil for immersion lenses is 1·515, in accordance with which such objectives are constructed.

An immersion fluid, similar to liquid paraffin, would nevertheless be a great boon on account of sundry disadvantages attaching to cedar oil. The latter is very often so thick that it impedes the microscopic examination of bacteria suspended in water or in watery media. It is frequently necessary to fix the coverslip in some way to avoid the disturbance caused by the moving up and down when focussing, which renders the observation of individual bacteria impossible. A limpid immersion fluid also which does not dry on the lenses and slides would be very acceptable.

Paraffin, which otherwise affords the required conditions, produces too great a deterioration of the microscopic image, as already mentioned.

After vainly searching for another liquid with a more suitable refractive index, it occurred to me that it might be possible to employ another substance with the same characteristics, but having a greater refractive index, which by mixing with paraffin might make it suitable.

My search led me to try *α*-bromnaphthalene, originally introduced into microscopy as an immersion fluid for use with specially constructed objectives. This is a thin liquid, almost colourless, and, so far as I know, harmless to lenses; while it has such a high boiling-point that inspissation does not occur at ordinary temperatures. Its refractive index is 1·656.

I prepared a mixture of bromnaphthalene and liquid paraffin which had the same refractive index as ordinary immersion oil. By experiment I found that about twenty-four parts of bromnaphthalene must be mixed with seventy-six parts of paraffin, but these quantities are only approximate, and in every case where greater accuracy is required the optimum mixture must be found experimentally by gradually adding the bromnaphthalene very carefully towards the end, as a single drop makes a distinct difference. It is much better if one can measure the refractive index with a refractometer, but a glass rod moved about in the fluid can also be used, the outline of which disappears when the correct mixture is obtained. Still better than the ordinary liquid paraffin is the American preparation "Nujol," which is thicker in consistency, and only requires the addition of about 12 per cent. bromnaphthalene to adjust it to the correct refractive index.

The mixture does not possess, in all respects, the same optical properties as genuine immersion oil, its dispersion particularly is somewhat different, but this is not observed in ordinary microscopic work. Even when using apochromatic objectives one has to observe very carefully and use the finest tests to detect any difference.

I have now used this fluid daily for about four years with immersion lenses of Zeiss, Leitz and Seibert, achromatic and apochromatic, and have always been satisfied with it. We have used it in our laboratory classes with great success, as students are far from careful with the microscopes placed at their disposal. They seldom return the stopper to the bottle of immersion oil, nor do they, as a rule, clean the lenses and slides. Also, from want of practice, it is difficult for them to see the form of bacteria when the latter are subjected to the slightest movement. Moreover, when used with preparations without a coverglass this immersion fluid has not the slightest effect even on such delicate stains as Romanowsky's (Leishman's, Giemsa's, etc.). It may be allowed to remain on the slide for months without any fading of the stain being observed. It is very easily removed from fresh or old preparations, whether it has been just put on the slide or whether it has been there a long time; it does not dry up. If one cannot wipe it away with a cloth for fear of injuring the specimen, it may be easily washed away with xylol.

In my opinion it has only one objection—it smells a little of bromnaphthalene, but it is really not a disagreeable odour.

To sum up the advantages of this fluid—it is thin in consistency, remains unchanged for months even in an open vessel, it does not dry on the slides or lenses, it does not influence the most delicate staining, and it is cheap to use.

It can be obtained from G. T. Gurr, 35, Perrymead Street, London, S.W. 6.

THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE NATURE AND SYSTEMATIC POSITION OF *B. PARATYPHOSUS C.*

F. W. ANDREWES AND SHEFFIELD NEAVE.*

From the Laboratories of St. Bartholomew's Hospital, London.

Received for publication May 4th, 1921.

DURING the war there were a number of outbreaks of infective disease in Eastern Europe and in Asia which were traced to a bacillus of the paratyphoid group apparently distinct from *B. paratyphosus A* and *B*, though having some affinities with the latter. The name *B. paratyphosus C* has been employed for this organism, and the evidence connecting it with the disease is of the same validity as in the case of the other paratyphoids—that is to say, the organism is readily recoverable by blood culture in those infected and it is present after death in the spleen or other viscera, while the serum of the patient exhibits characteristic agglutination of the organism. The symptoms produced by infection have been varied. At times they have resembled a paratyphoid fever of short course, in other cases they have been those of acute gastro-enteritis or dysentery, in others again the phenomena have been those of an acute septic condition, while in some they have been chiefly pulmonary, taking the form of bronchitis and pneumonia. Intestinal ulceration has been sometimes present, but often absent, and the bacillus has seldom been cultivated from the stools, though not infrequently from the urine. The mortality has been high in some outbreaks, low in others. Two non-fatal cases are known to have occurred in England since the war: one, in a little girl at St. Thomas's Hospital in 1920, has been published by Dudgeon(1); the other, in a man returned from Palestine, occurred at St. Bartholomew's Hospital in 1919 and is described in this paper. It was the starting-point of the investigation into the nature of the organism the results of which are here set forth.

The patient, a clerk, aged 25 years, served from March, 1917, to February, 1919, in the Royal Garrison Artillery in Palestine, and in no other field of the war. In April, 1918, he had an attack of amœbic dysentery which yielded to emetin. Later in the same year he was for one week in hospital in Alexandria with unexplained pyrexia, but quickly recovered and rejoined his unit in October, 1918. From that time till his return home he remained well. He left Palestine in February, 1919, in a transport crowded with soldiers returning from Mesopotamia. On reaching England in March he was perfectly well, and after two weeks' furlough resumed his work as a clerk in the London General Omnibus Company.

---

* Owing to Dr. Neave's absence from this country I have had to undertake the writing of this paper, and must assume responsibility for its deficiencies. Dr. Neave has helped me in the work throughout and his name should be associated with it.—F. W. A.

On May 3rd he had an illness diagnosed as influenza, and three days later this was complicated by pneumonia. He began to get up on May 15th, but had a rigor on May 18th, and on May 21st had diarrhœa lasting three days. He remained somewhat febrile, with occasional rigors, and on June 9th was admitted to St. Bartholomew's Hospital under the care of Dr. Drysdale. He was very ill, with irregular fever and rigors; there were no abdominal symptoms, but obscure signs at the base of one lung, which ultimately proved due to a small pleural effusion. Blood culture on June 23rd yielded in two tubes out of six a bacillus culturally resembling *B. paratyphosus B*, but inagglutinable with paratyphoid B serum. A culture sent to the Lister Institute was at once identified by Dr. Schütze as identical with the Bagdad strains of paratyphoid C. On June 30th the agglutinating powers of the patient's blood were investigated, using Dreyer's method; he had been inoculated with T.A.B. vaccine rather less than a year previously. The result was as follows:

B. typhosus 1 : 400 = 32 Dreyer units.
B. paratyphosus A, 1 : 164 = 39 Dreyer units.
B. paratyphosus B, 1 : 656 = 187 Dreyer units.
B. Aertrycke (Newport), 1 : 267.
,, (Mutton), 1 : 300.
Gärtner's bacillus, 0.
Patient's own bacillus, 1 : 1600.

A week later the titre to the patient's own bacillus had fallen to 1 in 1000, and that for T. A. and B. was practically unchanged, B showing a slight fall from 187 to 178 units. The fæces were negative on bacteriolcgical examination. On July 10th the urine was examined: it contained a moderate amount of pus and yielded an abundant and pure growth of a bacillus identical with that obtained from the blood, except that it was agglutinated by paratyphoid B serum to about half titre. The patient was put on urotropin and the pus and bacilli disappeared from the urine in a few days.

The man recovered; the fever ceased about the end of June; there was some femoral thrombosis early in July. After he left the hospital he had an attack of appendicitis, but when seen again in November he was quite well, and his urine was normal and negative on culture.

HISTORICAL.

The term "*B. paratyphosus* C" was proposed in 1908 by Uhlenhuth and Hübener(2) for a race of bacilli encountered by them in pigs in the course of their investigations on swine fever. They stated that it was culturally indistinguishable from *B. suipestifer*, but inagglutinable with sera of the hog cholera group and by Gärtner serum, while its own serum was equally without influence on hog cholera bacilli. They found the organism in the organs of pigs suffering from swine fever, in the stools of healthy men, pigs and calves, and in sausages, and they further identified it with a certain group of the organisms concerned in calf-dysentery. In connection with these statements it must be remembered that the Uhlenhuth school draws no sharp distinction between *B. suipestifer* and *B. paratyphosus B*, and regards the Aertrycke strains as practically identical with the latter organism; further, it places little reliance on the absorption test in serological work. There is, nevertheless, some evidence in the work of Dienes and Wagner, to be mentioned below, to suggest the identity of one, at least, of Uhlenhuth's strains with the organism we are about to consider. Outbreaks of disease due to members of the paratyphoid group inagglutinable with ordinary sera have since been observed from time to time in Germany. Heimann,(3) for example, has described an outbreak of meat-poisoning at Hildesheim in 1911, due to pork, in which the bacillus was of this type, but as he did not employ for his fermentation tests the substances now known to be of service in distinguishing between paratyphoid B and C and suipestifer, and as he did not test his bacillus against a suipestifer serum, it is impossible to be sure of the nature of the organism.

In 1914, or earlier, at Peshawur in India, a bacillus was isolated which Schütze(4) has tested and identified with *B. paratyphosus C*.

In 1915 Neukirch,(5) attached to the Turkish army in Armenia, encountered a considerable outbreak of disease due to an organism which he called the "Erzindjan bacillus." Cultures sent to Germany were there identified by means of "Voldagsen" and "Glässer" sera (of which more anon) with the suipestifer group.

In 1916 the same organism was observed in several of the fields of war. Weil and Saxl(6) describe three cases in Russian prisoners in Volhynia, and Weil(7) later had two further cases in Albania. Weil compared his bacillus with that of Neukirch and found it identical. Dienes and Wagner,(8) at Lemberg, investigated a large number of cases, mostly in Russian prisoners, and found bacilli agglutinating with "Voldagsen" serum, as well as a race apparently differing from *B. paratyphosus C*. They applied agglutination and absorption tests, and, happening to have a strain of Uhlenhuth's paratyphoid C from Krål's laboratory, they found that this absorbed the specific agglutinin from the sera made with their own strains. This is an important piece of evidence connecting the recent war strains with Uhlenhuth's organism. Dienes and Wagner affirm the identity of their bacilli of Group I with Weil's Volhynian strains and Neukirch's Erzindjan bacillus as well as with Uhlenhuth's paratyphoid C.

Meanwhile Hirschfeld,(9) bacteriologist to the Serbian army, had met with a disease resembling enteric, but due to a special bacillus which he called *Paratyphoid C*. He added this organism to the protective vaccine he was using. Dudgeon(1) also, in Macedonia, was in communication with Hirschfeld and observed cases of his own. He has done experimental work with the bacillus, which seems quite identical with that found by the other observers named.

Quite independently British workers in Mesopotamia met with the same bacillus in cases at Bagdad. These have been described by Mackie and

Bowen(10) and by MacAdam,(11) and cultures of these strains were brought back to England by Ledingham. Garrow(12) has also reported a case of paratyphoid C from East Africa.

These various findings may be regarded as establishing a type of paratyphoid infection of variable clinical character, due to a bacillus distinct from *B. paratyphosus A* and *B*, but having a certain serological relation with the latter. Many observers have found that while some paratyphoid C strains fail to react at all with a paratyphoid B serum, others are agglutinated by it to a quarter or a half of its full titre ; it has been observed also that a strain may change in this respect while under cultivation, a bacillus, at first incapable of agglutinating with paratyphoid B serum, gradually aquiring that property ; Hirschfeld reports a change in the opposite direction. More than this, it was found by Neukirch that when the bacillus was repeatedly cultivated from a single case, sometimes it reacted to some degree with paratyphoid B serum and sometimes not. Its agglutination with its own serum does not appear to vary.

CHARACTERS OF *B. VOLDAGSEN* AND *B. TYPHI SUIS* (GLÄSSER).

In the course of our study of the paratyphoid C bacillus we have compared it with a wide range of members of the Salmonella group, employing not only agglutination, but the absorption test. The organisms with which we have worked have been as follows :

B. typhosus, several strains ; *B. paratyphosus A*, several strains ; *B. paratyphosus B*, 8 or 10 strains ; *B. Aertrycke* (Newport race), 2 strains ; *B. Aertrycke* (Mutton race), several strains ; *B. paratyphosus* C, Hirschfeld's Serbian strain and 2 from Bagdad ; *B. Gärtner*, several strains ; *B. suipestifer*, 8 or more strains, chiefly American and German ; *B. Voldagsen* ; *B. typhi suis* of Glässer, 2 strains.

We owe many thanks to the various workers who have supplied us with these strains ; above all to the Lister Institute and to Prof. Jensen of Copenhagen, who kindly sent a supply of German strains, chiefly from Uhlenhuth's laboratory. We made our own sera from rabbits, but some were sent us from elsewhere. The following sera were employed :

B. typhosus, *B. paratyphosus A*, *B. Gärtner*. *B. paratyphosus B :* 2 of our own make and 1 from Oxford. *B. Aertrycke :* Newport and Mutton strains. *B. paratyphosus C :* 3 sera, viz. from the Hirschfeld strain and from the blood and urine strains of our own case. *B. suipestifer* : 3 sera, made respectively from English, American and German strains. *B. Voldagsen* and *B. Glässer*.

Inasmuch as *B. Voldagsen* and *B. typhi suis* (Glässer) played a part in the identification of *B. paratyphosus C* by the German observers, and as these organisms have been little if at all studied in England, it may be well to state some facts about them.

B. suipestifer, Voldagsen, was described by Dammann and Stedefeder (13) in 1910 as the cause of a disease in young pigs resembling swine-fever, and known as "Ferkel typhus" or "sucking-pig typhoid." Considerable dispute has arisen about it in Germany: the Uhlenhuth school maintain that it is a secondary invader, and that the primary disease is due to the usual filter-passer of swine-fever—a position denied by Pfeiler. These disputes need not concern us here; the interest lies in the fact that the Voldagsen bacillus differs considerably from *B. suipestifer* in its cultural characters, while evidently allied serologically.

B. typhi suis was described by Glässer (14) as similarly a cause of disease in pigs, and is by many regarded as identical with the Voldagsen bacillus. Like that organism it differs culturally from *B. suipestifer*, but resembles it in serological characters.

By the kindness of Prof. Jensen we received a culture of *B. Voldagsen*, of German origin, and two Glässer cultures, one emanating from Prof. Pfeiler's laboratory at Bromberg and one from Prof. Poppe, of Berlin. When these strains were plated out on agar they were seen to be much less vigorous in their growth than ordinary suipestifer strains; the colonies of *B. Voldagsen* in particular were minute, irregular in shape and very transparent, the Glässer colonies more regular and a little larger. Both organisms were actively motile and longer and thinner than *B. suipestifer*. The fermentative reactions were almost the same in the two types: no gas was formed in any sugar, or only very minute traces, except that one of the Glässer strains gave gas in xylose. Mannite was scarcely attacked; one Glässer strain would not ferment it at all, the other took 13 days and the Voldagsen strain 15 days to produce acid. The Glässer strains were similarly shy in attacking dulcite, taking 15 and 19 days to produce acid; the Voldagsen bacillus took only 4 days. All three strains, unlike *B. suipestifer*, readily attacked arabinose. Instead of producing an alkaline reaction in litmus milk, that fluid, after an initial acidity, became neutral again; even after 46 days there was no alkaline reaction, except for a faint trace in the case of *B. Voldagsen*. It will be seen that these characters differ so widely from those of *B. suipestifer* and the paratyphoids that one would be inclined to reckon the Voldagsen and Glässer bacilli as specifically distinct.

We prepared rabbit sera from the Voldagsen strain (titre about 50,000) and from one of the Glässer strains (titre about 20,000), and in numerous experiments we tested the agglutinating powers of these sera upon other Salmonellas, and the effect of our other Salmonella sera upon the Voldagsen and Glässer strains. Formalinised broth cultures of standard opacity were used by the macroscopic method, the tubes being partially immersed for two hours in a water-bath at 54° C. The Voldagsen serum agglutinated ordinary suipestifers to its full titre, and paratyphoid C strains to about half titre: paratyphoid B strains and Aertryckes were only weakly agglutinated and the Glässer strains scarcely at all. The Glässer serum agglutinated paratyphoid C strains and some suipestifers to titre or nearly so, the Voldagsen bacillus only weakly and the paratyphoid B strains and Aertryckes scarcely at all. Somewhat similar relations were found when Voldagsen and Glässer strains were exposed to the action of the various other sera. It was apparent that the Voldagsen and Glässer strains were by no means serologically identical, and this opinion was confirmed by absorption tests, in which it was found that a Voldagsen serum was completely exhausted of all agglutinins by saturation with the Glässer bacillus, while the specific agglutinin in the Glässer serum was almost unaffected by saturation with the Voldagsen bacillus.

It is thus manifest that in the suipestifer group serological and cultural characters do not necessarily go hand in hand.

One further remark may be made here, though it is not germane to the subject of the paratyphoid C bacillus. Owing chiefly to the work of Bainbridge (^{15}), it has become customary in this country to identify the Aertrycke bacillus with *B. suipestifer*. While it is clear from Bainbridge's careful work that the strains of Aertrycke and suipestifer which he used were serologically identical, we are inclined to believe that the strains of suipestifer supplied to him from Germany, where they use the term more loosely than we do, were not identical with the true hog cholera bacillus of Salmon and Smith. It is more likely that his suipestifer was in error than his Aertrycke strain, for we have obtained from Prof. Hutchens, of Newcastle, a culture believed to have descended from the strain of Aertrycke Bainbridge used, and this, though now poorly agglutinable, can still definitely be identified with the "Mutton" type of Aertrycke. Our own observations, extending over a considerable range of Aertryckes and suipestifers, indicate constant cultural and serological differences which forbid their identification. The Aertrycke strains are very much more closely allied to *B. paratyphosus B* than to *B. suipestifer*, though they appear to contain a trace of suipestifer antigen absent in *B. paratyphosus B*.

THE CULTURAL AND FERMENTATIVE CHARACTERS OF *B. PARATYPHOSUS C* COMPARED WITH THOSE OF OTHER SALMONELLAS.

All the members of the group agree in being motile bacilli, negative to Gram's stain, not liquefying gelatin and forming no indol. All fail to ferment lactose and saccharose: there are, however, four sugars or allied substances which enable us to effect a certain amount of differentiation amongst them; they are xylose, arabinose, dulcite and inosite. We have tested the reactions of seventy or more strains of known serological properties and our results confirm those of other observers. They are shown in the following table:

	Xylose.	Arabinose.	Dulcite.	Inosite.
B. paratyphosus A	−	+	+	−
,, *B*	+	+	+	+
,, *C*	+	+	+	−
B. Aertrycke (Newport)	+	+	+	−
,, (Mutton)	+	+	+	+
B. Gärtner	+	+	+	−
B. columbensis	+	+	+	−
B. suipestifer	+	−	−	−

The only exceptions we have found to the reactions shown in this table were in one strain out of eight of the Mutton type of Aertrycke and one out of twelve paratyphoid B's tested which failed to ferment inosite, and in three strains of suipestifer, two of which gave a late and imperfect reaction with dulcite and the other an initial transient acidity in arabinose. Dulcite and arabinose fermenting strains of *B. suipestifer* are known to occur or to arise by mutation, but the bulk of our strains were constant in refusing to attack these substances, and we failed to produce an arabinose fermenting mutant when two single bacillus strains were cultivated for several generations on arabinose agar plates.

It is well known that the various Salmonella strains mentioned above speedily produce an alkaline reaction in litmus milk; only *B. paratyphosus A* fails to do this, the alkaline reaction being delayed for several weeks. *B. paratyphosus C* forms alkali rather slowly. We adopted also another method of determining the rate of alkali formation in this group and its allies, testing some seventy strains. An ovo-mucin medium was tinted with phenol red and its reaction was so adjusted that after the final sterilisation it was P_H 6. The tubes were inoculated with one loopful of a 24-hour broth culture of each strain tested, and compared from day to day with a standard series of phenol red tubes ranging from P_H 6·6 to P_H 8. The time taken by the inoculated tubes to reach P_H 8 varied somewhat even with the same race; the figures obtained for this limit were as follows:

B. coli communis, 2 days; *B. columbensis*, 2 days; *B. Gärtner*, 3 to 6 days; *B. paratyphosus B*, 3, but sometimes 6 days; *B. Aertrycke*, 3 up to 6 days; *B. suipestifer*, 3 to 8 days; *B. paratyphosus C*, 8 to 10 days; *B. typhosus*, 8 to 13 days; *B. Voldagsen* and *B. Glässer*, 10 to 13 days; *B. paratyphosus A*, 13 days.

The figure for *B. fæcalis alkaligenes* could not be determined because it would not grow at P_H 6.

The various races were also roughly tested as regards production of hydrogen sulphide by growing them in broth tubes with basic lead acetate. *B. typhosus*, *B. paratyphosus A*, and *B. columbensis* gave practically no blackening, and there was not much in the case of *B. Voldagsen* and the Glässer strains; the remainder formed H_2S readily, the paratyphoid C's equally with the rest. Like most other Salmonellas the paratyphoid C's reduce neutral red, with fluorescence.

B. paratyphosus C is thus seen to be distinguished from *B. paratyphosus B* and the Mutton type of *B. Aertrycke* by its inability to ferment inosite and its slower rate of alkali production. It is culturally indistinguishable from Gärtner's bacillus and the Newport type of *B. Aertrycke*, except by its slower alkali formation. It differs from *B. suipestifer* in readily fermenting dulcite and arabinose. These are perhaps not sufficiently weighty reasons for regarding it as a distinct race if it were not for its peculiar serological characters, to which we will now turn.

THE SEROLOGICAL CHARACTERS OF *B. PARATYPHOSUS C.*

The inquiry upon which we have been engaged has extended over the various races of the Salmonellas pathogenic for man, and cannot fully be discussed here; but the relations of *B. paratyphosus C* to *B. suipestifer* render it necessary to give a short account of our observations on the latter organism in its serological aspects, limiting ourselves to the property of agglutination.

The puzzling relationships of the Salmonella group become a little clearer when search is made for particular antigenic components. The criterion of a specific antigen, *quâ* agglutination, is that it reacts only with the specific agglutinin which it evokes, and that this specific agglutinin cannot be absorbed by other antigens. We fully recognise that the doctrine of multiple antigenic components is no more than a working hypothesis, but it is necessary to postulate something of the sort if we are to offer any sort of explanation of the phenomena observed.

We have to consider three antigenic components in discussing *B. paratyphosus C.* Two of them belong essentially to *B. suipestifer* and the third to *B. paratyphosus B.* The reasons for assuming two suipestifer antigens are as follows: We have worked with nine suipestifer strains of porcine origin, apart from the Voldagsen and Glässer races. Now when these strains are submitted to agglutination by a Glässer serum, or the serum which we made from the *blood* strain of paratyphoid C from our own case, they are sharply differentiated into two groups. Either they are agglutinated to the full titre of the serum, or only to a weak degree (3 to 10 per cent. of the full titre).

Group I (agglutinated to full titre by the sera named above).

Swine fever (Royal Veterinary College); hog cholera (Arkansas); type suipestifer (No. 42), (Indiana); *B. typhi suis* (Glässer), (both our strains); paratyphoid C (all strains tested).

Group II (agglutinated very weakly by the sera named above).

B. suipestifer (Künzendorf strain); *B. suipestifer* (strains 327, 338, 339), (Uhlenhuth); *B. suipestifer* N_2 (isolated at the Lister Institute by Schütze); "intermediate 202" (Indiana); *B. Voldagsen.*

This differentiation is not sharply effected by any of the other Group I sera we have employed, not even by that of the urine strain of paratyphoid C from our case. Sera of Group II—*e. g.* a Voldagsen or suipestifer Künzendorf serum—agglutinate Groups I and II almost equally, except that they bring down the paratyphoid C's only to half titre, and the Glässer strains hardly at all.

The absorption test emphasises the distinction between the two groups of *B. suipestifer.* A member of either group will completely, or almost completely, remove all agglutinins from a serum belonging to its own group. But whereas a member of Group I will exhaust a Group II serum, a member of Group II fails to produce much effect in reducing the titre of a Group I serum, except for members of Group II. The following example of reciprocal absorptions between *B. Voldagsen* (Group II) and *B. Glässer* (Group I) will serve to illustrate this:

A. *Voldagsen Serum absorbed with* B. Glässer (*dose 5½ billion bacilli per c.c. undiluted serum*).

Strains used as test.	Titre of unabsorbed control.	Titre after absorption.
Aertrycke: Newport	7,700	0 (at 1 in 125).
Paratyphoid B	2,300	0
Paratyphoid C (Hirschfeld)	40,000	0
Hog cholera (Arkansas)	66,000	0
Suipestifer (Künzendorf)	68,000	0
B. Voldagsen	70,000	0
B. Glässer	200	0

B. *Glässer Serum absorbed with* B. Voldagsen (*dose* 10 *billion per c.c. serum*).

Strains used as test.	Titre of unabsorbed control.	Titre after absorption.
Paratyphoid C (Hirschfeld)	17,000	15,000
Hog cholera (Arkansas)	10,000	10,000
B. Glässer	17,000	17,000
Suipestifer (Künzendorf)	1,175	0 (at 1 in 125).
Suipestifer 339 (Uhlenhuth)	1,525	0
B. Voldagsen	1,100	0

It is not a little remarkable that these two races, showing similar cultural differences from other suipestifers, should exhibit the serological dissimilarities which divide the suipestifers themselves into two groups. It is also remarkable that the Glässer bacillus, though agglutinated to such a trifling degree by the Voldagsen serum, nevertheless effects such complete absorption.

The only explanation which we can frame for these facts is to imagine two suipestifer antigens, which we may call 1 and 2, and to suppose that Group I contains both these, while Group II contains only one.

The antigenic structure of the suipestifers is further complicated by the intrusion of a paratyphoid B element to a variable extent in different races. The evidence for this is derived both from agglutination and absorption. *B. Glässer* alone seems quite free from this element, and we believe that *B. paratyphosus* B (at least in its human type) is equally free from any suipestifer element. A paratyphoid B serum will not agglutinate *B. Glässer* at all, nor will a Glässer serum agglutinate *B. paratyphosus B*: no absorption of the specific agglutinin is witnessed in either serum on saturation with the other bacillus, however large the dose employed. One of our paratyphoid C strains, that isolated from the blood of our own case, was also, at first, almost equally free from the paratyphoid B element, its serum behaving like a Glässer serum; but under cultivation the paratyphoid B element gradually asserted itself, as we shall show later. The strain isolated from the urine of the same case contained from the first a large paratyphoid B element. In other suipestifers and in *B. Voldagsen* the paratyphoid B antigen is present in varying degree, as testified by slight or moderate mutual agglutination and absorption.

Turning now from this digression as to the grouping of the *B. suipestifer* strains to our observations on *B. paratyphosus C* itself, we will deal only with our own strains, isolated respectively from the blood and urine of the case described above, and with the Serbian Army strain from Hirschfeld, which we have used for purposes of comparison. We will speak of these as the Witts' blood strain, the Witts' urine strain and the Hirschfeld strain.

So far as their relations *inter se* are concerned, these three strains appeared identical. Apart from the fact that the Witts' blood strain was uniformly less agglutinable with all sera (by some 50 per cent.) than the other two, it may be said that the serum of any one of the three agglutinated the other two to full titre. Further, any one of the three completely exhausted the sera of the other two of primary and secondary agglutinins alike. A single example of this will suffice:

Serum: Witts' Urine Strain absorbed with Hirschfeld Strain (dose, 7 billion per c.c. Undiluted Serum).

Strains used in test.	Titres of unabsorbed control.	Titres after absorption.
Aertrycke: Newport	5,500	0 (at 1:125)
Aertrycke: Mutton	5,600	0
Paratyphoid B	7,600	0
Hirschfeld strain	11,000	0
Witts' urine strain	11,000	0
Swine fever	10,000	0
B. Voldagsen	8,500	0
B. Glässer	2,600	0

The reciprocal absorption gave quite similar results. We found that these paratyphoid C sera required very large doses to effect complete absorption; the Hirschfeld serum showed peculiar obstinacy in this respect. Thus, in absorptions of Hirschfeld serum with its own bacillus, a dose of 300,000 million per c.c. of serum produced little or no effect; a dose of 5 billion caused complete exhaustion.

When, on the other hand, we come to examine the behaviour of the paratyphoid C bacilli and their sera in relation to other members of the Salmonella group, a difference amongst them becomes apparent. As we shall show, our Witts' urine strain shows quite a marked relation with *B. paratyphosus B*, the Hirschfeld strain a less marked one and the Witts' blood strain almost none at all. Hence, in endeavouring to arrive at any conclusion as to the nature of *B. paratyphosus C*, it will be best to consider the last-named first, as presenting the "C" characters in their purest form.

A. *Serological Properties of the "Pure" Paratyphoid C (Witts' Blood Strain, when first Isolated).*

As regards agglutination and agglutinogenesis, the results shown in the following two tables indicate the remoteness of this strain from the paratyphoid B–Aertrycke group, and its clear relation to the *B. suipestifer* group. The figures given represent *percentage* agglutination, the titre of each serum for its own bacillus being taken as 100. They represent the averages of the figures obtained in many observations. The relatively poor agglutinability of Witt's blood strain makes the percentage figures for itself low, and those for other bacilli agglutinated by its serum high. Similarly the high agglutinability of the Witt's urine strain has precisely the opposite effect.

The first column in these tables on p. 167 shows that the "pure" paratyphoid C is not agglutinated at all by paratyphoid B and Aertrycke sera, and that its serum scarcely agglutinates the bacilli in question. On the other hand its relations with the suipestifer group, and notably with *B. Glässer*, are plain; but with Group II of the suipestifers (exemplified in the tables by *B. suipestifer* [Künzendorf] and *B. Voldagsen*) the relation is slighter, these bacilli being very feebly agglutinated by the Witts' blood strain serum.

The relations suggested by agglutination and agglutinogenesis are fully confirmed by mutual absorption tests, as we will now proceed to show. We have conducted nineteen quantitative absorption tests in which the Witts' blood strain

Percentage Agglutination of Paratyphoid C Strains by Salmonella Sera.

Sera.	Witts' blood strain.	Witts' urine strain.	Hirschfeld strain.
Aertrycke: Newport	0	30	39
Aertrycke: Mutton	0	33	46
Paratyphoid B	0	43	42
Paratyphoid C: Hirschfeld	56	92	100
Paratyphoid C: Witts' urine	45	100	100+
Paratyphoid C: Witts' blood	100	100+	100+
Swine fever (English)	37	52	57
Hog cholera (American)	28	30	47
B. suipestifer (Künzendorf)	31	45	50
B. Voldagsen	42	47	59
B. Glässer	82	100+	86

Percentage Agglutination of Various Salmonellas by Three Paratyphoid C Sera.

Strains tested.	Witts' blood strain serum.	Witts' urine strain serum.	Hirschfeld serum.
Aertrycke: Newport	1·5	56	5
Aertrycke: Mutton	1·7	58	7
Paratyphoid B	1·7	69	8
Paratyphoid C: Hirschfeld	100+	100+	100
Paratyphoid C: Witts' urine	100+	100	92
Paratyphoid C: Witts' blood	100	45	56
Swine fever (English)	100+	100+	100
Hog cholera (American)	100+	100+	100+
B. suipestifer (Künzendorf)	3	92	100
B. Voldagsen	3	99	86
B. Glässer	100+	29	67

NOTE.—The symbol 100+ indicates that, owing to differences in agglutinability, the titre was higher than that shown for the strain with which the serum had been prepared.

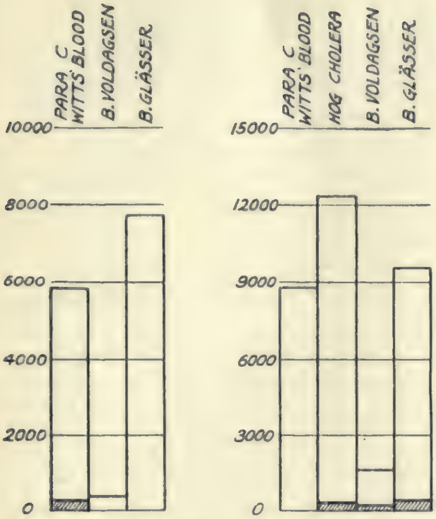
was used to absorb other sera, or in which its serum was absorbed with other Salmonellas: most of these have been reciprocal absorptions. The broad general results have been as follows: The paratyphoid C serum was exhausted of its specific agglutinin (more or less completely according to the dose of the absorbing organism) by other paratyphoid C's, by *B. Glässer* and, not quite so completely, by other members of suipestifer Group I (swine fever, Roy. Vet. Coll., and suipestifer 49 from America). The specific titre was not reduced at all in absorptions with suipestifers of Group II, with paratyphoid B, or with Aertrycke (Mutton and Newport). The specific agglutinin behaved in fact like a single entity: either it was quite unaffected or it was almost wholly absorbed. The serum used throughout was one made shortly after the isolation of the bacillus, and represented a "pure" paratyphoid C serum, but when we turn to the effects of the bacillus as an absorbing strain it must be remembered that, in the course of eighteen months, the organism gradually underwent mutation, finally approximating to the "urine strain." We could not, therefore, avoid the fallacy that we were operating with a less and less "pure" paratyphoid C strain. With this proviso, it may be stated that the Witts' blood strain paratyphoid C was able to effect an almost complete exhaustion, when used in adequate dose, of other paratyphoid C sera, of Glässer serum, and of all suipestifer sera both Group I and Group II: the latter fact definitely affiliates the strain with Group I of the suipestifers. Contrary to expectation, paratyphoid B and Aertrycke sera were found to be definitely reduced in their specific titre by saturation with this strain of paratyphoid C, the reduction amounting to one half or even more. Within two months of its isolation, and before any mutation was observed, this strain reduced the titre of a paratyphoid B serum from 3600 to 2100. At a later date, after mutation had begun, it reduced the titre of a Mutton serum to 46 per cent. and of a Newport serum to 25 per cent. of the initial value. These facts are difficult of explanation, but suggest some paratyphoid B element, latent or actual, in the paratyphoid C strain under consideration.

We have sought for a compact diagrammatic method of expressing our absorption results and have adopted that seen below. The heights of the columns represent the actual and not the percentage titres of the serum, before absorption, for the different bacterial races. The shaded areas indicate the several titres left after the absorption; the unshaded areas represent the actual agglutinin absorbed. Gross inequalities in the titres of different sera are corrected by altering the scale to which they are drawn. Four examples of reciprocal absorptions have been selected for reproduction. When the columns of both members of a pair are left unshaded, or nearly so, complete homology is suggested between the strains concerned: if both were shaded total heterology would be suggested, but this is seldom the case. When one member of a pair shows shaded and the other unshaded columns, the fact indicates a one-sided absorption relationship such as subsists between Groups I and II of the suipestifers. Dosage of the absorbing strain is given in billions (*i. e.* million millions) per c.c. of undiluted serum. Doses below one billion have often proved insufficient to effect maximal absorption: doses of two billion and upwards have almost always proved sufficient to absorb all the agglutinin which can be absorbed.

These diagrams may be left to speak for themselves. It will suffice to summarise the evidence we have obtained as to the serological relationships of the "pure" paratyphoid C represented by the Witts' blood strain.

(1) In spite of the striking differences in cultural characters, its serological identity with *B. Glässer* (which, again, is serologically a member of Group I of the suipestifers) is almost quite complete. Maximal absorption of a Glässer serum with the paratyphoid C left only 3 per cent. of the Glässer agglutinin: the converse absorption left only 2 per cent. of the paratyphoid C agglutinin, while the Berlin strain of *B. Glässer* absorbed the paratyphoid C serum completely. Further evidence of serological identity is afforded by the facts that (*a*) the serum of each, allowing for differences in agglutinability, brings down the other to full titre; (*b*) in the most varied absorptions of other sera with other Salmonellas the titres for these two organisms invariably behave alike; (*c*) they exhibit the same one-sided relationship to members of the Group II of *B. suipestifer*.

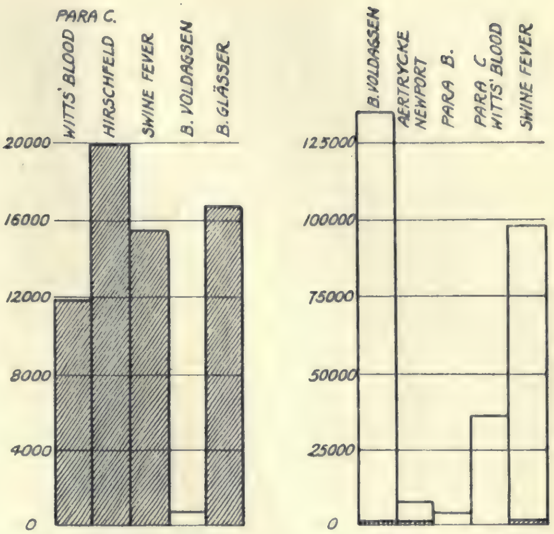
(2) The relations with other members of Group I of the suipestifers are



WITTS' BLOOD PARA C. SERUM ABSORBED WITH B. GLÄSSER. (DOSE 2·7 BILLION)

GLÄSSER SERUM ABSORBED WITH WITTS' BLOOD PARA C. (DOSE 6·5 BILLION)

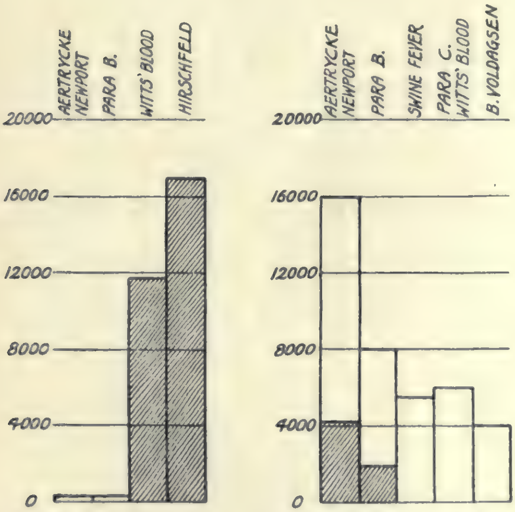
FIG. 1. RECIPROCAL ABSORPTIONS BETWEEN PARA C. (WITTS' BLOOD STRAIN) & B. GLÄSSER



WITTS' BLOOD PARA C. SERUM ABSORBED WITH B. VOLDAGSEN (DOSE 4·5 BILLION)

VOLDAGSEN SERUM ABSORBED WITH WITTS' BLOOD PARA C (DOSE 5 4 BILLION)

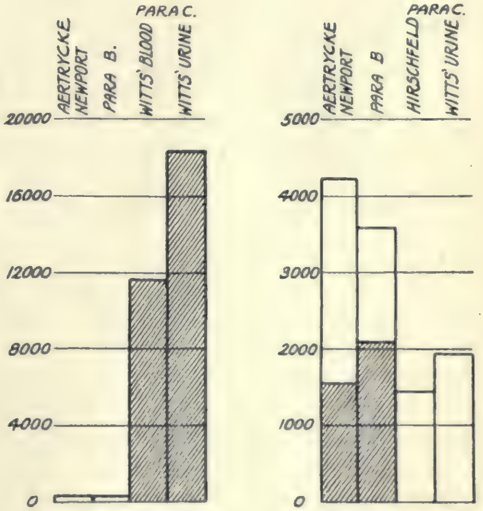
FIG. 2. RECIPROCAL ABSORPTIONS BETWEEN PARA C. (WITTS' BLOOD STRAIN) & B. VOLDAGSEN



WITTS' BLOOD PARA C. SERUM ABSORBED WITH B. AERTRYCKE (NEWPORT) (DOSE 1¼ BILLION)

AERTRYCKE (NEWPORT) SERUM ABSORBED WITH WITTS' BLOOD PARA C. (DOSE 7 BILLION)

FIG. 3. RECIPROCAL ABSORPTIONS BETWEEN PARATYPHOID C. (WITTS' BLOOD STRAIN) & B. AERTRYCKE (NEWPORT).



WITTS' BLOOD PARA C. SERUM ABSORBED WITH B. PARATYPHOSUS B. (DOSE 0·7 BILLION)

PARATYPHOID B. SERUM ABSORBED WITH WITTS' BLOOD PARA C. (DOSE 0·7 BILLION)

FIG. 4. RECIPROCAL ABSORPTIONS BETWEEN PARATYPHOID C. (WITTS' BLOOD STRAIN) & PARATYPHOID B.

almost as close as with *B. Glässer*, though here again there are definite differences as regards the sugars fermented. As seen in the tables given on a previous page, the "pure" paratyphoid C serum brings down swine fever (hog cholera) strains to titre, and the sera of the latter have a very definite agglutinating power on paratyphoid C—really more than is indicated in the table because of the low agglutinability of the Witts' blood strain and the very high agglutinability of the suipestifer strains in question. Group I suipestifers will absorb nearly all the specific agglutinin from the paratyphoid C serum, only 3 to 4 per cent. being left. The reciprocal experiment was not carried out with this strain, but the Witts' urine stain absorbed swine fever serum well, leaving, in two experiments, only 8 per cent. and 4 per cent. of the specific agglutinin.

(3) With Group II of the suipestifers the relations are characteristic of Group I. Group II sera agglutinate the paratyphoid C fairly well; the paratyphoid C serum has but a feeble agglutinating power on members of Group II. Group II sera are exhausted of all their specific agglutinin on absorption with Witts' blood strain; but the pure paratyphoid C serum is wholly unaffected by large doses of Group II suipestifers (up to 4½ billion).

(4) As regards relationship with paratyphoid B and the Aertrycke bacilli, they are very slight in the case of this strain. Mutual agglutination with their sera is absent or very weak. The specific titre of the serum of Witts blood strain is unaffected by very heavy saturation with paratyphoid B or *B. Aertrycke;* nevertheless, a potential relationship is suggested by the fact that Witts' blood strain even when first isolated did effect a distinct reduction of specific titre in a paratyphoid B serum, though this serum would not agglutinate it to any measurable degree. The potential relationship becomes an actual one when mutation occurs.

(5) No other relationships have been discovered. We have tested a Gärtner serum, a paratyphoid A serum and a typhoid serum on our paratyphoid C strains, and our paratyphoid C sera on these bacilli, with wholly negative result.

B. *Serological Properties of the Ordinary Strains of Paratyphoid C.*

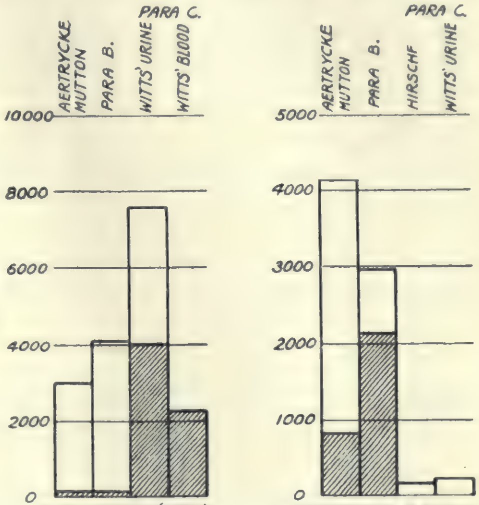
The remaining strains of paratyphoid C which we have examined, representing what appears to be the more usual type, closely resemble the preceding in all respects, but differ in the intrusion of a paratyphoid B element to a variable extent, which leads to certain differences in serological behaviour. This shows itself in the following ways:

(*a*) These ordinary paratyphoid C strains are agglutinated by a paratyphoid B serum to something like a half of its full titre; they are similarly agglutinated by Aertrycke sera.

(*b*) Their sera agglutinate paratyphoid B and *B. Aertrycke* to a degree varying in its extent. Our paratyphoid C (Hirschfeld strain) serum acted only weakly on these bacilli, but the serum of our Witts' urine strain paratyphoid C agglutinated them to half or even two-thirds of its full titre.

(*c*) In absorption experiments there is seen evidence of a closer relation between these ordinary paratyphoid C's and the paratyphoid B-Aertrycke group than was the case with the "pure" paratyphoid C strain. This is illustrated in the following graph, which represents reciprocal absorptions between *B. paratyphosus B* and the Witts' urine strain of *B. paratyphosus C.*

It is seen that this paratyphoid C strain removes about a quarter of the specific agglutinin from the paratyphoid B serum, and that the paratyphoid B strain removes half the specific agglutinin from the paratyphoid C serum. It is further of interest to note that whereas this is the case with the titre of the C serum for its homologous bacillus, the titre for the "pure" paratyphoid C (Witts' blood strain) is not affected at all by the absorption with paratyphoid B. This phenomenon was constantly observed in absorptions of Witts' urine strain serum by paratyphoid B or by Aertrycke strains. It may be effectively represented by a graph of a different kind showing the *percentage* reductions in its titres for different strains suffered by this paratyphoid C serum (Witts' urine strain) when absorbed with three different doses of paratyphoid B (fig. 6).



PARA C. SERUM (WITTS' URINE STRAIN) ABSORBED WITH PARATYPHOID B. (DOSE 5 BILLION)

PARATYPHOID B SERUM ABSORBED WITH PARA C. (WITTS' URINE STRAIN) (DOSE 1·2 BILLION)

FIG. 5. RECIPROCAL ABSORPTIONS BETWEEN PARATYPHOID C. (WITTS' URINE STRAIN) & PARATYPHOID B.

It is seen that the medium dose brings down the titre for the "compound" paratyphoid C's by some 50 per cent., no further reduction being effected by the very large dose; the titre for the "pure" paratyphoid C remains unaltered; that for paratyphoid B and *B. Aertrycke* is practically extinguished. One would be tempted to surmise that when such a compound paratyphoid C strain is agglutinated by its own serum, the effect is a composite one due to both C and B agglutinins, only the latter being removed by the absorption with paratyphoid B. We are, however, by no means disposed to argue from this single serum that all sera agglutinate in composite fashion by the resultant action of different components, for many facts are opposed to such a view.

It may be added that our "Witts' urine strain" of paratyphoid C appeared to contain a much larger paratyphoid B element than the Hirschfeld strain.

Only one further difference was detected between the pure paratyphoid C and the strains showing a paratyphoid B element. Whereas the serum of the former showed no reduction in its specific titre when absorbed with Group II suipestifers, the serum of our Witts' urine strain of paratyphoid C suffered a reduction of more than half, sometimes of more than three-quarters of its specific titre when so absorbed. There was never complete absorption. Group I suipestifers were able to absorb the agglutinin from this serum fairly completely, and the Hirschfeld serum was absolutely exhausted by *B. Glässer.*

THE MUTATION OF THE PURE INTO THE COMPOSITE *PARATYPHOID C.*

This phenomenon has been observed by most of those who have worked with this organism and we had the opportunity of following it in our own case. It has been explained that the strains isolated from the blood and from the urine differed in their relation to paratyphoid B. The urine strain was from the first agglutinated by a paratyphoid B serum to rather less than half titre, while paratyphoid B was agglutinated by the serum of this strain to $\frac{2}{3}$ of the titre of the latter for itself. No change was observed in the urine strain under 18 months' cultivation.

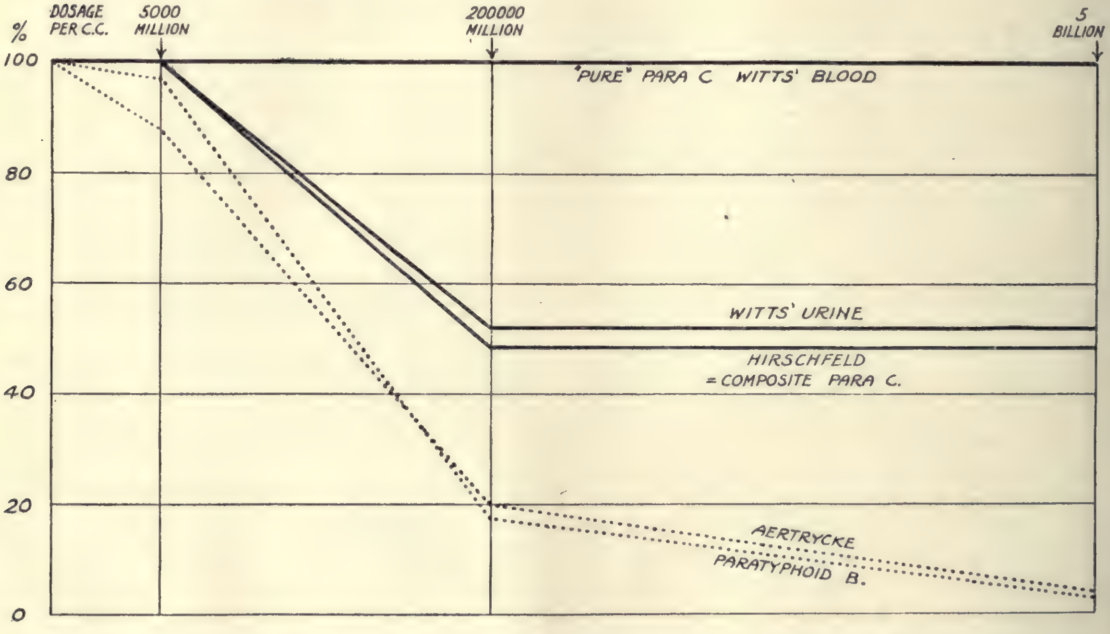


FIG. 6. PARATYPHOID C SERUM (WITTS' URINE) ABSORBED WITH 3 DIFFERENT DOSES OF B. PARATYPHOSUS B. SHEWING CURVES OF PERCENTAGE REDUCTION IN TITRE FOR DIFFERENT STRAINS.

It was otherwise with the blood strain. On first isolation it failed to agglutinate at all with a paratyphoid B serum (titre 1 : 5000) at all dilutions from 1 : 12½ upwards, and it was equally inagglutinable with Aertrycke sera. The strain was not inherently in-agglutinable, for it clumped freely with suitable sera. Our practice was to make a litre or so of formalinised broth culture and use this for all experiments ; when it was used up a fresh stock was made, the culture having been kept up on egg-medium (only occasionally sub-cultured). Stocks of emulsion were made in July and December, 1919, and in July, 1920. In using the December stock it was noted that the strain showed some degree of agglutination with paratyphoid B and Aertrycke sera, and this was still more pronounced with the stock made in July, 1920. As we had some of all three stocks of emulsion available a comparison was made between them—all being put up simultaneously against sera of paratyphoid B and the Newport and Mutton types of *B. Aertrycke.* The readings were as follows ;

Serum.	Emulsion 1. Made July, 1919, shortly after isolation.	Emulsion 2. Made Dec., 1919, after 5 mths'. culture.	Emulsion 3. Made July, 1920, after 12 mths'. culture.	Control with homologous bacillus.
Paratyphoid B.	Traces only	6,100 = 32 %	10,600 = 55 %	Para. B = 19,000
Aertrycke : Newport	„	2,210 = 13 %	6,100 = 38 %	Newport = 16,000
„ Mutton	„	512 = 12 %	1,235 = 28 %	Mutton = 4,400

It is seen that a progressive increase of agglutinability has occurred, most marked with the paratyphoid B serum. In the course of 12 months' subculture the blood-strain of our paratyphoid C has become serologically indistinguishable from the urine strain. Much is of course made of such facts as these by those who distrust serological classification, but it must be noted that this mutation of *B. paratyphosus C* involves no change in its essential specific agglutination ; it is a side issue, involving only a co-agglutinin. Further, it is not a haphazard change, but an orderly and regular one seen by all who have had the opportunity of working with a "pure" paratyphoid C strain. The change is always in the same direction and does not pass a certain limit—that is to say, the C strain becomes agglutinable by a paratyphoid B serum to about 50 per cent. of the latter's full titre and not more; it does not become transformed into a paratyphoid B. Hirschfeld states that he has witnessed a reverse change, a strain of paratyphoid C, at first partially agglutinable with a paratyphoid serum, finally losing that property ; this is the only instance of this which we have found recorded in the literature of the subject.

CONCLUSIONS.

(1) A new form of paratyphoid fever has been observed during the war by many investigators, chiefly in Eastern Europe and in Asia. The causal organism, *B. paratyphosus C*, is distinct from *B. paratyphosus A* and *B*, though presenting relationship to the latter.

(2) In this paper there is described a case of paratyphoid C occurring in England. The bacillus isolated is compared with other members of the Salmonella group in detail.

(3) In its cultural and fermentative characters *B. paratyphosus C* is shown to differ from *B. paratyphosus B* in its failure to ferment inosite and in its slower rate of alkali production. It differs from *B. suipestifer* in fermenting arabinose and dulcite.

(4) A short review is given of the serological properties of *B. suipestifer* and of the related forms *B. Voldagsen* and *B. Glässer*. We find the suipestifers to fall into two serological groups, sharply separable by certain sera. By absorption tests it is shown that a Group I suipestifer will exhaust the sera of both Groups I and II, but that a Group II suipestifer will exhaust only Group II sera, leaving the specific titre of Group I sera untouched.

(5) *B. paratyphosus C* is shown to belong serologically to the Group I suipestifers, in spite of the divergence in cultural characters.

(6) Different strains of *B. paratyphosus C* vary in their relation to *B. paratyphosus B*. In some the B element is absent, in others very obvious. An example of each variety was isolated from the case described, and it is shown that the strain which at first showed no relation to *B. paratyphosus B* gradually underwent a serological change, acquiring the property of being partially agglutinated by a paratyphoid B serum.

NOTE.

We do not make any claim to priority in affirming the relation of *B. paratyphosus C* to *B. suipestifer.* It was the knowledge that it could be agglutinated by Voldagsen and Glässer sera which induced us to compare it in detail with *B. suipestifer.* The division of the suipestifers into two serological groups is, we believe, novel; it enables us to associate *B. paratyphosus C* with one special group of suipestifers.

After the greater part of our work had been completed we read the paper of Dr. Carl Tenbroeck ([16]) on "Bacilli of the Hog-Cholera Group in Man." Tenbroeck had received two strains of *B. paratyphosus C* from Hirschfeld, and found on comparing them with *B. suipestifer* that there was strong serological correspondence, with complete mutual absorption. He adds a further piece of evidence: two rabbits immunised against Hirschfeld's paratyphoid C strains were found to be protected against a fatal dose of virulent hog-cholera bacilli.

REFERENCES.

1. Dudgeon, L. S., and Urquhart, A. L.—"The Paratyphoid 'C' Bacillus as a Cause of Paratyphoid Fever," *Lancet,* 1920, **2**, 15.
2. Uhlenhuth and Hübener.—"Weitere Mitteilungen über Schweinepest mit besonderer Berücksichtigung der Bakteriologie der Hogcholeragruppe," *Centralbl. f. Bakteriol.*, 1909, **42**, (Ref.) Beiheft, p. 127.
3. Heimann, W.—"Ueber die durch einen sogenannten 'Paratyphus C' Bacillus verursachte Fleischvergiftungsepidemie in Hildesheim im Frühjahr, 1911," *ibid.* 1912, **66** (Orig.) 211.
4. Schütze, H.—"The Paratyphoid B Group," *Lancet,* 1920, **1**, 93.
5. Neukirch, P.—"Ueber Paratyphusbakterien im Blute bei ruhrähnlichen Erkrankungen in der Türkei," *Berl. klin. Wochenschr.*, 1917, **54**, 360. "Ueber menschliche Erkrankungen durch Bazillen der Glässer-Voldagsen-gruppe in der Türkei," *Ztschr. f. Hyg.*, 1918, **85**, 103.
6. Weil, E., and Saxl, P.—"Ueber eine Infektionskrankheit, bedingt durch einen Keim aus der Paratyphusgruppe," *Wien. klin. Wochenschr.*, 1917, **30**, 519.
7. Weil, E. — "Paratyphus B-ähnliche Krankheitserreger (Typus Suipestifer Voldagsen) in Albanien," *ibid.*, 1917, **30**, 1061.
8. Dienes, L., and Wagner, R.—"Ueber Paratyphus B-Infektionen," *Ztschr. f. Hyg.*, 1918, **87**, 157.
9. Hirschfeld, L.—"A New Germ of Paratyphoid," *Lancet,* 1919, **1**, 296.
10. Mackie, F. P., and Bowen, G. J.—"Note on the Characters of an Anomalous Member of the Paratyphoid Group met with in Mesopotamia," *J. R. Army Med. Corps,* 1919, **33**, 154.
11. MacAdam, W.—"An Account of an Infection in Mesopotamia due to a Bacillus of the Gaertner-Paratyphoid Group," *Lancet,* 1919, **2**, 189.
12. Garrow, R. P.—"A Case of Paratyphoid C Fever in East Africa," *ibid.*, 1920, **1**, 1221.
13. Dammann and Stedefeder.—"Untersuchungen über Schweinepest," *Arch. f. wiss. u. prakt. Tierheilk.*, 1910, **36**, 432.
14. Glässer, K.—*Deutsch. tierärztl. Wochenschr.*, 1909, pp. 513, 529. Ref. *Centralbl. f. Bakteriol.*, 1910, **45**; (Ref.), 612.
15. Bainbridge, F. A.—"The Differentiation of the Paratyphoid and 'Food-poisoning' Bacilli," *J. Path. and Bacteriol.*, 1908–9, **13**, 443, 465.
16. Tenbroeck, C.—"Bacilli of the Hog-Cholera Group (Bacillus choleræ suis) in Man," *J. Exper. Med.*, 1920, **32**, 33.

ON SOME CHARACTERS OF THE CLEAVAGE PRODUCTS OF CERTAIN BACTERIA, WITH SPECIAL REFERENCE TO THEIR TOXICITY AND ANTIGENIC PROPERTIES.

S. R. DOUGLAS, M.R.C.S. (Capt. I.M.S., retired),
Acting Director, Bacteriological Department, National Institute for Medical Research, Hampstead, London.

Received for publication June 15th, 1921.

UP to the present time the production of antibacterial sera, with perhaps the exception of that made from *B. dysenteriæ* (Shiga), has proved very difficult, and the sera, when made, have usually been found to give unsatisfactory results.

In making such sera the usual procedure is to inoculate horses with increasing doses of emulsions of either living or dead bacteria, but there is no generally recognised method used in their preparation or in testing their potency. For the latter purpose estimation of one or other of the so-called antibodies (such as the agglutinating, opsonic or bactericidal) has usually been employed ; an example of this is the titration of the agglutinating power of antimeningococcus serum which is supposed to be a means of measuring its content of antitoxin.

Although the estimation of any of the antibodies probably gives some information concerning the degree of immunity attained in response to a series of inoculations, the very general belief that the antibodies are all formed together and that the estimation of one gives a comparative measurement of all, is not true. For the knowledge of the order and intensity in which antibodies are formed is still very incomplete, as is our knowledge of the nature and formation of the so-called endotoxins. This being the case, it appeared to be of fundamental importance to study these problems in any way possible.

In order to follow the *raison d'être* of the following experiments it will be necessary to consider what sequence of events follows on the inoculation of a bacterial vaccine, or a sublethal dose of living bacilli. Suppose, for instance, a dose of 500 million dead typhoid bacilli is injected under the skin of a man, it is known that shortly after the injection a considerable outpouring of lymph occurs at the site of inoculation, and that even in a previously uninoculated man this lymph has a considerable power of breaking down the bacteria and completely dissolving them. The solution of the dissolved bacteria is carried away in the lymph-stream, and if rapidly absorbed, as is the case when the inoculated man or animal takes active exercise or in any way stimulates the lymph-flow, causes a certain series of well-recognised symptoms, such as fever, headache and general malaise. These symptoms rapidly subside, and, as has been frequently shown, are followed by an increased agglutinating, opsonic and lytic power of the blood-fluids, and under certain circumstances, far less well investigated, by protective power against the living organisms, and by the power to precipitate bacterial filtrates.

Taking these as the recognised facts, one essential for the production of the intoxication and general symptoms appears to be the lytic action of the blood-fluids which enables the broken-down bacteria to be carried in solution into the circulation, and it seems only reasonable to believe that unless the organisms of a vaccine are dissolved they cannot give rise to toxic symptoms, and that, only then, can antibodies be produced.

In a former research in conjunction with A. Fleming (1), it was shown that organisms which had been extracted with acetone were very readily attacked and dissolved by trypsin, while living suspensions of the same bacilli were little or not at all affected by this ferment, and it was further shown that these acetone-extracted organisms formed as good or even better vaccines as regards the production of antibodies than the unextracted bacilli killed by heat.

Another experiment showed that when typhoid bacilli were dissolved by means of normal human serum and the resulting mixture was centrifuged until a clear supernatant fluid was obtained, a perceptible precipitate was formed when this clear fluid was added to the serum of an animal that had received repeated doses of typhoid vaccines. This precipitin reaction was specific.

As a result of the conclusions drawn from these experiments, it was decided to ascertain the effect of inoculating acetone-extracted bacilli which had been acted on by trypsin, and also to examine such digested emulsions in respect of their toxicity and general antigenic properties.

THE EFFECT OF TRYPTIC DIGESTION ON THE TOXICITY OF BACTERIA.

Preparation of bacteria for digestion.—A series of agar cultures of a strain of *B. dysenteriæ* (Shiga) were grown in Roux bottles for 48 hours at 37° C. The growth was then washed off with just sufficient salt solution and the resultant emulsion divided into two parts. To both portions sufficient acetone was added, namely about 50 per cent., to bring about agglomeration and sedimentation of the bacteria. One portion was then placed in large tubes containing about 150 c.c. each and centrifuged until all the bacteria were caked into a mass at the bottom of the tube. The clear supernatant fluid was then decanted off, and the caked bacterial mass was spread over the sides of the tubes in an even, thin layer and afterwards placed in the incubator at 37° C. to dry. The dried scales were scraped off the sides of the tubes and preserved in a desiccator. The other portion of the emulsion was, after the addition of the acetone, allowed to sediment, and the supernatant fluid poured off as far as possible. Fresh pure acetone was then added, and when the organisms had again sedimented the supernatant acetone was removed. The concentrated sediment was then poured into a Soxhlet thimble, and, when the acetone had filtered away, the thimble containing the bacterial mass was placed in a Soxhlet apparatus and extracted with acetone for about forty hours. The contents of the thimble were then dried at 37° C. in the incubator and these acetone-extracted organisms stored in powdered form in tubes. Preliminary experiments were then made comparing the toxicity of equal weights of the dried and acetone-extracted organisms. No difference could be detected, 0·075 mgrm. of each preparation killing rabbits of 1000 grm.

Method for preparing the digests.—Emulsions of these acetone-extracted bacilli were made in salt solution, each c.c. containing 10 mgrm. Two c.c. of such an emulsion were then placed in a dwarf test-tube and 100 c.mm. of a 1 per cent. sodium carbonate solution and 10 c.mm. of a sterile trypsin solution were added for each c.c., together with two or three drops of ether to prevent bacterial growth, and the tubes were sealed. Digestion was allowed to take place for about 4 to 18 hours at 37° C. As a result of the action of the trypsin the emulsion became almost completely clarified. In some experiments 10 per cent. of trypsin has been employed, but the results were almost identical.

The relation between the toxicity of the digests and the time of digestion.—This clear, or almost clear fluid was then tested for toxicity and it was found to kill rabbits of about 1000 grm. weight in doses representing 0·075 mgrm. of the acetone-extracted bacilli, when the digestion had been carried on for about eight hours at 37° C. The time that the trypsin is allowed to act appears to be an important factor. Numerous experiments were made and the following conclusions arrived at:

(1) Emulsions digested for short periods, such as 1 to 2 hours at 37° C. with 1 per cent. of trypsin, are not as toxic as an equal amount of the undigested emulsion.

(2) Emulsions digested for longer periods under similar conditions—for instance, 18 to 24 hours in the case of the strain employed in the experiments mentioned above—are also less toxic than the undigested emulsion.

(3) Different strains of *B. dysenteriæ* (Shiga) required different times of digestion to obtain the maximum toxicity, the optimum with the strain used in the above experiment being 8 to 10 hours at 37° C.

The relation between the toxin present in solutions of digested bacilli and that in suspensions of undigested bacilli.—The toxin obtained by digesting such emulsions gave rise to exactly the same symptoms as were produced by undigested bacilli, and the post-mortem appearances were also identical. To ascertain if the toxin, present in the digests, was the same as that which was derived from the intact bacilli, experiments were made to ascertain if an antitoxin, made by injecting horses with emulsions of untreated bacilli, completely neutralised the toxin contained in the digests.

The strain of *B. dysenteriæ* (Shiga) used in these experiments was somewhat deficient in toxic properties, and, in consequence, larger doses than usual had to be employed.

Experiment 1.

In the first experiment 5 rabbits, each about 1000 to 1250 grm., were used. Into No. 1, 0·5 mgrm. of an emulsion of acetone-extracted bacilli, which had been digested for about 4 hours at 50° C. with 10 per cent. trypsin, was injected intravenously. No. 2 was injected with 1·0 mgrm. treated in a similar manner. No. 3 received 5 mgrm., digested in the same manner, mixed with

0·5 c.c. of antitoxic serum three-quarters of an hour before the injection. No. 4 received 10 mgrm. treated in the same way, mixed with 1 c.c. of the same antitoxic serum. This mixture was also incubated for three-quarters of an hour at 37° C. before injection. No. 5 received 1 mgrm. of the acetone-extracted bacilli, which had *not* been digested.

The serum was obtained from Messrs. Burroughs Wellcome & Co., and was from a horse which had received injections of *B. dysenteriæ* (Shiga) only. The results of this experiment, which was carried out on October 4th, 1920, are shown in the following tables:

No. of rabbit.	Substances injected.	Result.
No. 1	0·5 mgrm. digested acetone-extracted bacilli	Partially paralysed on 8.10.20 and very nearly died, then slowly recovered.
No. 2	1·0 mgrm. digested	Died 8.10.20. Post mortem, typical lesions.
No. 3	5 mgrm. digested + 0·5 c.c. antitoxic serum	Died on 5.10.20. Post mortem, coccidiosis and tape-worm cysts found; rabbit very thin.
No. 4	10 mgrm. digested + 1 c.c. antitoxic serum	Remained quite well.
No. 5	1 mgrm. undigested acetone-extracted bacilli	Ill for 3 or 4 days, then recovered.

Experiment 2.

Six rabbits, each about 1000 to 1250 grm. in weight. Injections made on October 14th, 1920. Digestion of acetone-extracted bacilli carried on for 4 hours at 50° C. with 10 per cent. trypsin. All injections made intravenously. Results are shown in the following table:

No. of rabbit.	Substances injected.	Result.
No. 1	0·5 mgrm. digested bacilli	Died on 17.10.20. Post mortem, typical lesions.
No. 2	1·0 mgrm. digested bacilli	Died on 16.10.20. Post mortem, typical lesions.
No. 3	5 mgrm. digested bacilli, 0·5 c.c. antitoxic serum	Remained well.
No. 4	10 mgrm. digested bacilli, 1 c.c. antitoxic serum	Remained well.
No. 5	1 mgrm. acetone-extracted bacilli, undigested	Died 18.10.20. Post mortem, typical lesions.
No. 6	2 mgrm. acetone-extracted bacilli, undigested	Very ill for 2 or 3 days, but recovered.

The toxin and antitoxin mixtures were incubated for about one and a half hours at 37° C. before injection. This last experiment brings out two points: The first is that when digested for exactly the right time the digests of this strain are more toxic than the untreated bacilli. The second is that multiple lethal doses of untreated bacilli sometimes fail to kill; this may be explained by the body-fluids, etc., failing to break down the bacteria into the toxic "phase."

As already mentioned, these experiments were carried out with *B. dysenteriæ* (Shiga), but numerous experiments were also made with a strain of *B. typhosus* with very similar results; thus dried and acetone-extracted bacilli killed mice when similar quantities of the organisms (namely 1 mgrm.) were injected intra-peritoneally. The same emulsion of acetone-extracted bacilli digested for about 18 hours at 37° C., or for somewhat longer periods at room temperature with 10 per cent. trypsin, invariably killed in doses of 1 mgrm., and frequently in smaller doses such as 0·5 mgrm. or even less. The signs of disease produced during life and the post-mortem appearances were similar; the latter consisted of an enlarged, deeply-coloured spleen, congested adrenals and an extensive necrosis of the intestine.

Experiments made with acetone-extracted meningococci also showed that emulsions, after being digested with trypsin, had a considerable toxicity; for instance, doses of 0·1 mgrm. or even less were found to be fatal when injected subdurally into guinea-pigs, producing a typical and constant series of symptoms and giving rise to constant *post-mortem* changes. The full details of these experiments and methods for testing the antitoxic value of meningococcic sera, etc., form, however, a special research, which is being undertaken in conjunction with Dr. Colebrook.

On the question of the toxic substance being formed during the process of digestion.—On consideration of all the results obtained it would appear probable that the actual toxic substance is produced during the digestion of the organisms, whether by the body-fluids or by trypsin. If, however, the digestion is carried on for too long a period, this substance is further broken down and the solution becomes less toxic. As the cleavage must continue during practically the whole period of digestion, only a proportion of the whole available toxin that could be theoretically produced from a given quantity of the dried bacterial substance can be present in solution at any given time.

Amongst the facts pointing to the toxin being produced *in vitro* by breaking down the bacterial proteids by the action of the tryptic ferment, the following may be mentioned:

(i) The toxic solutions, however concentrated, only give opalescence on the addition of half their volume of 33 per cent. trichloracetic acid.

(ii) After the injection of digests of *B. dysenteriæ* or *B. typhosus*, even in sublethal doses, diarrhœa is produced in a very short time, such as 30 minutes, while when undigested bacteria are employed, in similar doses, no diarrhœa is noticed until after the lapse of a much longer period.

(iii) Different varieties of bacteria, and perhaps different strains of the same organisms, give the maximum toxicity after varying times of digestion.

(iv) From time to time it has been noticed when giving injections of dead bacilli that a certain dose will always kill the animal, but if a larger dose, perhaps two or three times as large as is necessary to kill the animal, is given, the animal survives. Such a fact has been recorded by Gordon (2) in the case of the meningococcus, and, in one of the experiments given in detail above, 2 mgrm. of dried *B. dysenteriæ* (Shiga) failed to kill, whereas, in this experiment and several others, 1 mgrm. proved to be the lethal dose for rabbits of the size used in the experiments.

The most satisfactory explanation of these facts is that when the dose surpasses a certain amount the animal is unable to break down the bacteria into the toxic "phase," and, in consequence, is able to survive a dose capable of killing several animals.

If the digests were poured into ten times their volume of alcohol a heavy precipitate was formed, which was easily collected by centrifuging. This precipitate which had remained in contact with the alcohol for from seven to ten days so as to coagulate all the free proteid present, and which had been washed free of some of the amino-acids and other substances by dialysis under pressure through a collodium membrane, was extracted with faintly alkaline salt solution, and this extract filtered was found to be still strongly toxic—in fact the alcoholic precipitate was much more toxic than an equal weight of the original dried bacilli. This shows that the toxin was not a proteid body capable of being coagulated by alcohol.

THE ANTIBODY RESPONSE TO INJECTIONS OF DIGESTED BACTERIA.

Experiments were carried out to amplify, and confirm, the information derived from a single experiment, the results of which were given in a paper written in conjunction with A. Fleming (1), in which the digested organisms were used as the antigen for inoculating animals, and to ascertain more exactly what response these animals made to such injections.

Experiment 1: Injection of Digested Bacilli.

In the first experiment, the details of which are given below, *B. typhosus* was the organism employed.

Six rabbits of about equal weights were taken; No. 1 and No. 2 were inoculated throughout the experiment with acetone-extracted bacilli; No. 3 and No. 4 with acetone-extracted bacilli which had been digested overnight with 10 per cent. trypsin at 37° C.; No. 5 and No. 6 with dried typhoid bacilli). All the inoculations were given intravenously.

On 31.12.20 the amount injected was 0·1 mgrm.

On 4.1.21, 0·3 mgrm. was inoculated.

On 13.1.21 a sample of blood was taken from each animal, and the agglutinating and bactericidal powers were estimated, with the following results:

Agglutinating Power of Sera.

No. of rabbit.	Dilutions of serum. 1 in:									
	25	50	100	200	400	800	1600	3200	6400	12,800
Rabbit No. 1	+	+	+	+	+	+	+	—	—	—
,, No. 2	+	+	+	+	+	+	+	S.	—	—
,, No. 3	—	—	—	—	—	—	—	—	—	—
,, No. 4	—	—	—	—	—	—	—	—	—	—
,, No. 5	+	+	+	+	+	+	+	+	+	S.—
Rabbit No. 6	+	+	+	+	+	+	+	S.+	—	—

In estimating the agglutinating power of the serum Dreyer's method was employed, and the same emulsion, a formolised broth culture suitably diluted, was used throughout the whole series of experiments.

Bactericidal Power (Wright's Method).

Dilutions of culture.	Control normal rabbit.	Rabbit No. 1.	Rabbit No. 2.	Rabbit No. 3.	Rabbit No. 4.	Rabbit No. 5.	Rabbit No. 6.
1 in 1,000,000	Growth	Growth	Sterile	Sterile	Sterile	Sterile	Growth.
1 in 100,000	,,	,,	Growth	,,	,,	Growth	,,
1 in 10,000	,,	,,	,,	,,	,,	,,	,,
1 in 1000	,,	,,	,,	,,	Growth	,,	,,
1 in 100	,,	,,	,,	Growth	,,	,,	,,
1 in 10	,,	,,	,,	,,	,,	,,	,,

Ten c.mm. of diluted culture mixed in each case with 10 c.mm. of serum (Wright's method). Culture contained 480 million living bacilli per c.c.

A further test was made on 17 . 1 . 21 of the agglutinating, bactericidal and opsonic powers of the serum of each animal.

Agglutinating Power.

No of rabbit.	Dilutions of serum. 1 in:											
	25	50	100	200	400	800	1600	3200	6400	12,800	25,000	50,000
No. 1	+	+	+	+	+	+	S.	—	—	—	—	—
,, 2	+	+	+	+	+	+	+	S.+	—	—	—	—
,, 3	S.—	—	—	—	—	—	—	—	—	—	—	—
,, 4	—	—	—	—	—	—	—	—	—	—	—	—
,, 5	+	+	+	+	+	+	+	+	S.+	—	—	—
,, 6	+	+	+	+	+	+	+	S.+	—	—	—	—

Bactericidal Power (Wright's Method).

Dilutions of culture.	Control normal rabbit.	Rabbit No. 1.	Rabbit No. 2.	Rabbit No. 3.	Rabbit No. 4.	Rabbit No. 5.	Rabbit No. 6.
1 in 1,000,000	Growth	Sterile	Sterile	Sterile	Sterile	Sterile	Sterile.
1 in 100,000	,,	Growth	,,	,,	,,	,,	Growth.
1 in 10,000	,,	,,	,,	,,	,,	,,	,,
1 in 1,000	,,	,,	Growth	,,	,,	Growth	,,
1 in 100	,,	,,	,,	,,	,,	,,	,,
1 in 10	,,	,,	,,	Growth	Growth	,,	,,

The culture contained 520 million living bacilli per c.c.

Opsonic Power.

The sera of rabbits Nos. 1, 2, 5 and 6 were all found to have very greatly increased opsonic power, while those of rabbits Nos. 3 and 4 remained almost within normal limits.

These results show that the inoculation of the acetone-extracted bacilli and dried bacilli produced practically identical results, namely an increase of the agglutinating, opsonic and bactericidal powers, while the inoculation of the digested acetone-extracted bacilli produced a very marked rise of the bactericidal power greater than did either the acetone-extracted or the dried bacilli, but *no* increase of the opsonic or agglutinating powers.

A further series of intravenous inoculation was administered to the same animals.

On 19.1.21, 0·5 mgrm. was inoculated.

On 24.1.21, 1·0 mgrm. was inoculated.

On 25.1.21, rabbit No. 1 died of gas gangrene.

On 28.1.21, 2·0 mgrm. were inoculated into the surviving five rabbits.

A sample of blood taken before this inoculation showed the agglutinating power of No. 2, No. 3 and No. 4 to be as follows:

Agglutinating Power.

	Dilutions of sera. 1 in:												
No. of rabbit.	25	50	100	200	400	800	1600	3200	6400	12,800	25,600	50,000	100,000
No. 2	+	+	+	+	+	+	+	+	+	+	S.—	—	—
„ 3	—	—	—	—	—	—	—	—	—	—	—	—	—
„ 4	—	—	—	—	—	—	—	—	—	—	—	—	—

The inoculations were continued.

On 31.1.21, 4·0 mgrm. were inoculated.

On 4.2.21, 8·0 mgrm. of the bacilli were injected.

A sample of blood was taken from rabbits No. 2, No. 3 and No. 4 before the inoculation, and the agglutinating and precipitating powers were tested.

Agglutinating Power.

	Dilutions of sera. 1 in:												
No. of rabbit.	25	50	100	200	400	800	1600	3200	6400	12,800	25,000	50,000	100,000
No. 2	+	+	+	+	+	+	+	+	+	+	S.+	—	—
„ 3	+	S.—	—	—	+	+	—	—	—	—	—	—	—
„ 4	Tr.	—	—	—	—	—	—	—	—	—	—	—	—

Precipitating Power.

In these experiments the antigen employed was the filtrate of a digested emulsion of acetone-extracted bacilli containing 5 mgrm. per c.c., the digestion being carried out at 37° C. with 1 per cent. trypsin overnight. One hundred c.mm. of serum was added in each case to 1·0 c.c. of the diluted antigen. The amount of precipitate formed is roughly indicated by the number of crosses.

	Dilutions of antigen. 1 in:					
No. of rabbit.	Undil.	2	4	8	16	32
No. 2	+++	++	+	Trace+	Trace—	Haze
No. 3	++	+++	+++	++	+	+
No. 4	++	+++	+++	++	+	+

These results indicate that the inoculation of a series of doses from 0·1 up to 4·0 mgrm. of the digested acetone-extracted bacilli produced practically no increase of the agglutinating power above the normal, while the precipitating power was increased considerably more than in the case of the rabbit which received the undigested acetone-extracted bacilli.

The inoculations were continued.

On 7.2.21, rabbits No. 2, No. 3, No. 4, No. 5 and No. 6 received doses of 16 mgrm.

On 14.2.21 a sample of blood was taken from each animal, and the agglutinating power was estimated with the following results :

Agglutinating Power.

No. of rabbit.	Dilutions of serum. 1 in :												
	25	50	100	200	400	800	1600	3200	6400	12,800	25,000	50,000	100,000
No. 2.	+	+	+	+	+	+	+	+	+	+	+	+	Tr.+
„ 3.	+	+	+	+	+	Tr.+	—	—	—	—	—	—	—
„ 4.	+	+	Tr.	—	—	—	—	—	—	—	—	—	—
„ 5.	+	+	+	+	+	+	+	+	+	+	+	+	S.+
„ 6.	+	+	+	+	+	+	+	+	+	+	+	+	S.+

Bactericidal Power (Wright's Method).

All the sera were found to be either normal or below normal in respect of the bactericidal power. It was noted that No. 5 and No. 6 had even less power than the normal animal.

Precipitating Power.

The same technique and antigen was used as in the former experiments.

No. of rabbit.	Dilutions of antigen. 1 in :					
	2	4	8	16	32	64
No. 2.	++	+	+	±	—	—
„ 3.	+++	+++	++	++	+	±
„ 4.	+++	+++	+++	++	+	Tr.
„ 5.	++	++	++	+	Tr.	—
„ 6.	++	++	++	+	+	—

The inoculations were continued.

On 18.2.21, 20 mgrm. were inoculated.

On 25.2.21, 20 mgrm. were inoculated.

On 25.2.21, No. 6 died.

On 4.3.21, samples of blood were taken from the surviving four rabbits, and the sera was tested for the agglutinating, opsonic and precipitating powers.

Agglutinating Power.

No. of rabbit.	Dilutions of serum. 1 in :												
	25	50	100	200	400	800	1600	3200	6400	12,800	25,000	50,000	100,000
No. 2.	+	+	+	+	+	+	+	+	+	+	+	+	—
„ 3.	+	+	+	+	+	+	—	—	—	—	—	—	—
„ 4.	+	+	+	+	+	+	S.	—	—	—	—	—	—
„ 5.	+	+	+	+	+	+	+	+	+	+	+	+	S.+

Opsonic Power.

By the dilution method No. 2 and No. 5 were found to be enormously increased, while No. 3 and No. 4 were almost within normal limits.

Precipitating Power.

The same antigen was employed, but to have the same quantitative measurement the technique consisted in adding 0·5 c.c. of the serum to 1 c.c. of the antigen in a tube drawn out to a contracted end. The precipitate was allowed to settle for 24 hours after being in a bath at 50° C. for 4 hours, and the tubes were centrifuged until the precipitate was tightly packed. The tube was marked at the upper border of the precipitate, then cleaned out thoroughly, dried and measured amounts of mercury run in until it occupied the same bulk as the precipitate.

The results were as follows:

Rabbit No. 2 gave 5 c.mm. of precipitate; rabbit No. 3, 10 c.mm.; rabbit No. 4, 8 c.mm., and rabbit No. 5 only a trace less than 2 c.mm.

These latter results show that a slight amount of agglutinating power had been produced in the serum of the animals inoculated with the digested acetone-extracted organisms. This was most probably due to the fact that a small percentage of the organisms escape digestion, so that when large doses, such as 20 mgrm., of the bacilli are injected, a sufficient number remain undigested to produce a comparatively slight increase in the agglutinating power.

The opsonic power of No. 3 and No. 4 remained almost normal, while the precipitating power of these two animals was increased considerably more than No. 2 or No. 5, which had received undigested organisms.

Summary of the Results of this Experiment.

It will be noted that in response to the earlier and smaller injections no increase of agglutinating power was developed in the sera of the animals injected with the digested organisms, but, when the larger doses of digested organisms were employed, some slight increase of the agglutinating power was produced. Almost exactly similar results were observed as regards the opsonic power, but the bactericidal power and precipitating power were largely increased.

Experiment 2.—Injection of Filtered Digested Bacilli.

Further experiments in which filtrates or thoroughly centrifuged digested emulsions of acetone-extracted bacilli were used as the antigen for intravenous injections showed that no increase of agglutinating or opsonic power was brought about, even after repeated injections, while the bactericidal and precipitating powers were much raised. The details of this experiment, in which *B. typhosus* was again employed, were as follows:

Three rabbits were taken, two of which—No. 1 and No. 3—had some months before received one or more doses of filtrates of digests of acetone-extracted bacilli. Rabbit No. 2 had never received any previous injections.

On 17.3.21, Nos. 1 and 2 received intravenously 0·1 c.c. of an emulsion of acetone-extracted typhoid bacilli, 5 mgrm. to 1 c.c., which had been digested overnight at 37° C. with 1 per cent. trypsin and then filtered through rough paper pulp.

On 18.3.21, No. 3 received 0·5 c.c. of the same filtrate.

On 21.3.21, No. 1 and No. 2 received 0·25 c.c., and No. 3, 0·5 c.c. intravenously of the same filtrate.

On 30.3.21, No. 1, No. 2 and No. 3 each received 0·5 c.c. intravenously.

On 2.4.21, No. 1, No. 4 and No. 3 each received 1 c.c. intravenously.

On 6.4.21, No. 1, No. 2 and No. 3 were given 2 c.c. intravenously.

On 9.4.21, No. 1, No. 2 and No. 3 were each inoculated intravenously with 1 c.c. of the filtrate, which had been concentrated to about one-fourth of its volume by filtration through a collodium membrane.

On 12.4.21, a sample bleeding was made.

None of the sera agglutinated a sensitive emulsion of *B. typhosus* in 1 in 25 dilution.

Precipitating power was well developed. A filtrate of a digested emulsion of acetone-extracted *B. typhosus* was used as the antigen.

	Dilutions of antigen. 1 in.:				
No. of rabbit.	2	4	8	16	32
No. 1.	++	++	+	Trace	—
,, 2.	++	+	Trace	—	—
,, 3.	++	++	+	+	Trace

Bactericidal Power.

Dilutions of culture.	Rabbit No. 1.	Rabbit No. 2.	Rabbit No. 3.	Control normal.
1 in 1,000,000	Sterile	Sterile	Sterile	Sterile
1 in 100,000	,,	,,	,,	,,
1 in 10,000	,,	,,	,,	Growth
1 in 1,000	Growth	,,	Growth	,,
1 in 100	,,	Growth	,,	,,
1 in 10	,,	,,	,,	,,

The inoculations were continued.

On 13.4.21, No. 1, No. 2 and No. 3 each were inoculated with 1·75 c.c. of the concentrated filtrate.

On 16.4.21, No. 1, No. 2 and No. 3 were inoculated intravenously with 1 c.c. of a filtrate which had been precipitated with alcohol and re-dissolved in saline.

On 22.4.21 a further sample bleeding.

Agglutinating power was absent from all the sera in 1 in 25 dilution.

Precipitating Power.

The precipitating power is shown below. The antigen employed was a solution of the digest which had been precipitated with alcohol and re-dissolved in saline.

	Dilutions of antigen. 1 in:					
No. of rabbit.	10	20	40	80	160	320
No. 1.	+++	+++	++	++	Trace +	Trace —
,, 2.	+++	++	+	Trace	—	—
,, 3.	+++	++	++	++	Trace	—

On 23.4.21, No. 1, No. 2 and No. 3 each were inoculated intravenously with 1 c.c. of the same solution as was given on 16.4.21.

On 29.4.21 a further sample bleeding.

Agglutinating Power.

Serum of No. 1 agglutinated to standard + in 1 to 2½ dilution, and gave a trace in the 1 in 5, but no signs of agglutination in 1 in 10 or higher dilutions.

No. 2 gave trace in 1 in 2½, but nothing in any higher dilution.

No. 3 gave standard + in 1 in 2½, nothing in the higher dilutions.

These agglutinating powers, it may be noted, fall well within the normal limits.

Precipitating Power.

Same antigen as in the last experiment.

	Dilutions of antigen. 1 in:					
No. of rabbit.	5	10	20	40	80	160
No. 1.	+++	+++	++	+	±	Tr. —
„ 2.	+++	++	+	±	—	—
„ 3.	+++	+++	++	+	±	Tr. —

On 4.5.21 a further sample bleeding was taken.

Agglutinating Power.

	Dilutions of antigen. 1 in:					
No. of rabbit.	2½	5	10	20	40	80
No. 1.	S.	—	—	—	—	—
„ 2.	S.+	—	—	—	—	—
„ 3.	S.+	—	—	—	—	—

Precipitating Power.

The same antigen was employed.

	Dilutions of antigen. 1 in:					
No. of rabbit.	10	20	40	80	160	320
No. 1.	+++	+++	++	+	Trace	—
„ 2.	+++	+++	++	+	„	—
„ 3.	+++	+++	++	+	„	—

To obtain a more accurate gauge of the precipitating power of these sera 0·5 c.c. of each serum was mixed with 1 c.c. of a digest, and after standing overnight the tubes were centrifuged and the volume of the precipitate measured by means of mercury.

It was found that No. 1 gave 11 c.mm. of precipitate, No. 2 gave 8 c.mm. and No. 3 gave 11 c.mm.

This experiment therefore shows that no agglutinins are formed when the digests are freed from undigested bacteria.

PRECIPITABLE SUBSTANCE PRESENT IN DIGESTS OF ACETONE-EXTRACTED BACTERIA.

While carrying out some of the above experiments it was observed that, on mixing the digests of organisms with the corresponding antitoxic serum a considerable precipitate was formed, and on making further tests this precipitate was found to be specific, and the phenomenon was, in fact, a typical specific precipitin reaction.

Further experiments showed that digests of not only *B. dysenteriæ*, but also of *B. typhosus*, meningococcus, tubercle bacilli and other organisms, filtered through paper pulp or a Berkfeld filter until perfectly clear, gave heavy precipitates on the addition of sera obtained from animals which had received repeated injections of the bacteria in question.

In addition to these results a large number of experiments were made in which human serum, after being digested until only a small amount of precipitate was formed on the addition of 33 per cent. trichloracetic acid, was mixed in various dilutions with a constant volume of the serum of a rabbit which had received several injections of unaltered human serum. A copious precipitate was formed with certain dilutions, but it was found that, when a comparison was made of digested and undigested serum, the undigested sample could always be diluted to a higher degree than the digested serum before the end-point of precipitation was reached. This appears unavoidable, as whatever substances produce the precipitate on being mixed with the immune serum, a proportion is destroyed owing to the length of time the digestion must be carried on, since, as in the case of the endotoxins, prolonged digestion leads to the destruction of the precipitable substance. When digests of bacteria are precipitated with alcohol for 7 to 10 days so as to coagulate any unaltered albumens, a filtered extract of this precipitate gives rise to a precipitate on the addition of the corresponding immune serum. In the case of digests of bacteria the precipitate formed was often abundant, and the extract of the alcoholic precipitate could be greatly diluted before an end-point was reached, but when a similar experiment was made using digested blood-serum the precipitate obtained was very much smaller in bulk, and was much more slowly formed than when unaltered serum was used in the test. Control experiments made by digesting serum obtained from other mammals showed this precipitate to be specific. The precipitates, whether they were obtained from a digest of bacteria mixed with the corresponding immune serum or with serum mixed with its appropriate antiserum, were constantly found to be insoluble in solutions of trypsin, even when washed free from all unaltered serum and suspended in alkaline salt solution. The significance of this fact will be discussed in a later section.

The production of the precipitating power in animals.—To ascertain if a specific precipitating power to albuminous fluids, such as blood-serum, was produced in the serum of rabbits, as the result of intravenous injection of serum, which had been digested until practically all the proteid constituents had been broken down by trypsin, rabbits were inoculated intravenously with human blood-serum, which had been digested with a large excess of trypsin until only a very slight precipitate was formed on the addition of 33 per cent. trichloracetic acid. After a rabbit had received two intravenous injections, representing 3 c.c. of serum in all, the animal produced a serum which had a power of producing the specific precipitate when mixed in the proportion of 100 c.mm. of this rabbit serum to 1 c.c. of diluted untreated human serum, up to a dilution of 1 in 1280.

After human blood-serum had been digested and the resulting solution had been precipitated with 10 volumes of alcohol the precipitate was extracted with saline and passed through an ordinary filter-paper. This clear solution, as has been shown above, when mixed in suitable amounts with anti-human rabbit's serum gave a distinct although slight precipitate. When, however, it was employed as the antigen and injected intravenously into a rabbit no development of precipitating power could be detected. The details of this experiment are given below:

The rabbit received intravenously first the re-dissolved precipitate from 1 c.c. of blood-serum, the next day that from 3 c.c., and 3 days later the remainder of the precipitate representing 6 c.c.—in all the precipitate from 10 c.c. of blood-serum. Sample bleedings were taken 6 and 10 days after the last injection, but no precipitating power could be demonstrated.

From the above experiments it appears probable that the precipitating power of the serum which is produced in response to injections of bacteria or serum, is brought about by the reaction of the body, *not* to the unaltered proteids of the bacteria or serum, but to some degradation product of these proteids which is produced by the action of the proteolytic ferments present in the blood-fluids when untreated bacilli are injected.

SIMILAR WORK CARRIED OUT BY OTHERS.

Experiments of somewhat similar character, resulting in various conclusions, have been carried out by other workers. For instance, R. Pfeiffer (3) maintained that the lytic action of the body-fluids caused the toxicity of several species of bacilli; Vaughan (4) held that the endotoxins were degradation products of the bacterial protoplasm; and many other workers have investigated the toxic properties of certain bacteria, usually *B. typhosus*, by breaking down the bacilli either by mechanical means (Macfaydean) (5), or by the lytic action of the serum (Besredka) (6). More recently Jobling, using ferments obtained from leucocytes (leuco-proteases), came to the conclusion in his early work (1912) (7) that the cleavage products of pneumococci were capable, when injected into animals, of giving rise to a specific opsonic increase, while in a later work (1913) (8), in which *B. typhosus* was the organism employed, he concluded that although the toxic substance which is produced when the bacilli are broken down with leucoprotease is the same as that which results from the breaking down of the bacilli by the lytic action of the blood-fluids, etc., *in vivo*, yet it is not the true endotoxin, since the same symptoms and lesions can be produced when other bacilli are employed. Quite recently (1920) Sanarelli (9), working with cultures of *V. choleræ* partially digested with trypsin, came to the conclusion that the specific endotoxin, which he believes is present in the intact vibrio, is set free during the digestion, and produces, when injected intravenously into guinea-pigs in sufficient quantity, a condition very similar to the disease seen in man. Further, he believes that these digested cultures form as complete an antigen as the undigested bacilli, and are capable of producing all the recognisable antibodies.

When these results and conclusions are contrasted with those obtained in the present research it is seen that in the case of *B. dysenteriæ* (Shiga) the toxin present in the digests is the specific endotoxin, and that this is also the case with meningococcus; here the specificity is so marked that some difference could be detected between the various types. The details of these experiments, which were carried out in conjunction with L. Colebrook, will be given in a later publication. As regards the digests forming as complete an antigen as the undigested organisms, this research shows conclusively that no increase of the agglutinating or opsonic powers is produced in response to injections of digested bacteria, if care is taken to exclude bacteria on which the ferment has not acted.

DISCUSSION.

On consideration of the various results it may be stated with a considerable degree of certainty that the agglutinating and precipitating powers of a serum, instead of being connected very closely with one another, are in reality widely separated, being in all probability produced in the body in response to very different stages of the cleavage of the bacteria proteids.

It would also appear probable that, when intact bacteria are injected into an animal, the agglutinating and opsonic powers are produced in response to a very early stage of the dissolution of the bacteria by the tissue fluids. At a later stage the lytic powers are developed ; a still more advanced stage of the same process produces the specific " endotoxin " to which the body reacts by the production of the antitoxin; lastly, and very nearly connected with this toxic substance, is that in response to which the precipitating power is developed. By the last reaction the cleavage products of the foreign proteid are returned to an insoluble phase which is incapable of being acted on by such ferments as trypsin, and most probably by those ferments present in the body-fluids, which appear to act in a similar manner. The removal of this precipitate from the circulation is possibly carried out by the leucocytes, which then settle in certain of the internal organs. Its final disposal is carried out in some manner at present unknown, but not improbably from the mucous surfaces by a process similar to that whereby the excess of lymphocytes is disposed of.

It appears advisable to point out some of the ways in which bacteria which have been broken down by trypsin may prove of practical value. Bacteria broken down to the toxic stage appear to be in the best form (i) for testing the antitoxic value of any antibacterial serum, since their use entirely overcomes the difficulty of employing multiple lethal doses, which, when the undigested bateria are employed, sometimes fail to kill. (ii) The toxic products may be considerably increased in potency by such methods as precipitation with alcohol, removal of the amino-acids, etc.—the details of which will be given later. They are therefore in a much better form for inoculating into animals for the production of antitoxin, and it is to be hoped that by their employment more efficient antitoxin may be obtained. (iii) In response to injections of the digests certain of the antibodies, which further experiments may increase in number, are not developed. It is therefore possible that the mechanism whereby the remaining antibodies are produced in the body will not be so easily exhausted, and, in any case, the bacterial proteid, having been broken down to the toxic stage outside the body, the disappearance of the lytic power as a result of numerous injections will not be of the same importance.

If it is true that—in accordance with the theory propounded above—the toxicity of some organisms is due to their proteid constituents being broken down by the proteolytic ferments of the body-fluids, it is obvious that, when these are rendered less active, as gauged by the diminished bacteriolytic power, the antitoxic power cannot be increased above a certain range. However many injections of bacilli may be given, very little or none of their proteid constituents will be presented to the body-cells and fluids in the "phase" suitable to produce an "antitoxic response"; this would also apply to the production of precipitating sera. If, however, before the injections are given, the proteids of the bacilli are broken down outside the body to the suitable "phase," there should be no limit to the range of the antitoxic and precipitating response, and an improvement in the production of anti-endotoxic sera may follow the use of these digests in immunisation.

Digests of bacteria form excellent antigens for employment in tests for complement-deviation, and by their use it may be possible to ascertain when certain chronic diseases are in an active condition, since when a bacterial infection is progressive some of the bacteria must come into contact with the body-fluids and cells. In consequence, degradation products of the bacterial proteids will be produced, and the body will develop an increase of the antibodies in response to these, which should be detected by complement-deviation tests in which a suitable digest is used for the antigen. If, however, the disease becomes arrested completely no bacteria will be acted on in this way, and therefore the antibody content of the blood against such cleavage products should fall to within normal limits.

Digests of bacteria may also prove of use as vaccines, especially therapeutically. The type of patient which may be benefited by their use is that which has become "tolerant" to the ordinary vaccine made by killing the bacteria by heat. Such subjects appear to be unable to break down the vaccine into the stage necessary for the development of antibodies, and might be greatly benefited by inoculation of suitably broken-down bacteria, especially where there is no evidence of autoinoculation.

I should like to thank Dr. W. Gye for kindly criticism, and Dr. L. Colebrook for carrying out several of the opsonic estimations.

SOME CONCLUSIONS.

Emulsions of acetone-extracted bacteria which have been digested with trypsin for a suitable period have a toxicity which is the same as, or at times greater than, that of emulsions of untreated bacteria of equal density.

The toxin present in the tryptic digests is the specific bacterial endotoxin which is usually produced in the body by the lytic action of the body-fluids.

Antitoxin prepared by the injection of untreated bacteria completely neutralises the toxic properties of the tryptic digests.

When bacterial digests are injected into animals the bactericidal and precipitating powers are greatly increased. Antitoxin is also formed, but there is no increase of either the agglutinating or opsonic powers.

This shows that the agglutinating and precipitating properties of the serum, instead of being very closely related or even identical, are quite distinct from one another.

It is also suggested that the antigens giving rise to the agglutinating and opsonic powers are produced during the very early stages of the solution of the bacteria by the body-fluids, while the antigens, in response to which the body produces the bactericidal, antitoxic and precipitating powers, are formed during a comparatively late period of the lytic process.

Repeated injections of untreated bacteria cause diminution of the lytic power of the blood-fluids even when comparatively small doses are employed. This would account for the difficulty in producing potent anti-endotoxic sera, as the loss of the lytic power would prevent the injected bacteria being digested *in vivo* as far as the toxic phase, and, in consequence, no antitoxic response would be evoked.

Digestion of the bacteria *in vitro* presents the endotoxin preformed to the body, and so may lead to the production of much more potent antitoxic sera.

Digests of bacteria should prove to be more suitable for testing the potency of anti-endotoxic sera than emulsions of untreated bacteria, and since such digests contain large amounts of the specific precipitable substance they should be employed in estimating the precipitating properties of sera.

Other tests in which such digests may be employed are:

(i) As antigens in complement-fixation tests; here it is suggested that the activity of a disease might be recognised by the detection in the blood of antibodies to particular products of bacterial cleavage by using as the antigen the corresponding phase of a tryptic digest.

(ii) As vaccines for therapeutic use, especially in those subjects which have become "tolerant" to the ordinary vaccine—a condition in which the body appears to have lost all power of breaking down the bacteria of the vaccine.

REFERENCES.

1. Douglas, S. R., and Fleming, A.—"On the Antigenic Properties of Acetone-extracted Bacteria," *Brit. J. Exper. Pathol.*, 1921, **2**, 131.
2. Gordon, M. H.—"Cerebro-spinal Fever," *Med. Res. Council, Spec. Rep. Ser.*, No. 50, 1920, p. 100 (H.M. Stationery Office).
3. Pfeiffer, R., and Kolle, W.—"Ueber die specifische Immunitätsreaction der Typhus Bacillen," *Ztschr. f. Hyg. u. Infektionskrankh.*, 1896, **21**, 203.
4. Vaughan, V. C.—"A Contribution to the Chemistry of the Bacterial Cell and a Study of the Effects of some of the Split Products on Animals. Part I: General Statement. Part II: The Bacterial Cell. Part III: The Production of Active Immunity with the Split Products of the Colon Bacillus," *Boston Med. and Surg. J.*, 1906, **155**, 215, 243 and 271.
5. Macfaydean, A., and Rowland, S.—"An Intracellular Toxin of the Typhoid Bacillus," *Proc. R. Soc.*, 1902–3, B, **71**, 77.
6. Besredka, A.—"Études sur le bacille typhique et le bacille de la peste," *Ann. Inst. Pasteur*, 1905, **19**, 477.
7. Jobling, J. W., and Strouse, S.—"Immunisation with Proteolytic Cleavage Products of Pneumococci," *J. Exper. Med.*, 1912, **16**, 860.
8. Jobling, J. W., and Bull, C. G.—"Toxic Split Products of *Bacillus typhosus*," *ibid.*, 1913, **17**, 453.
9. Sanarelli, G.—"Le protéide du Vibrion cholérique," *Ann. Inst. Pasteur*, 1920, **34**, 370.

THE OCCURRENCE OF HÆMOLYTIC SUBSTANCES IN NORMAL URINE.

ERIC PONDER.

From the Department of Physiology, Edinburgh University.

Received for publication February 16th, 1921.

In a paper recently published (¹) it was pointed out that certain specimens of normal urine contained hæmolytic substances. The number of normal cases examined, however, was small, and the cases were not unselected; the results, therefore, could not be taken as representative of the frequency with which hæmolytic substances occurred in normal urine. An opportunity of examining a large number of specimens of the urine of normal persons has since occurred; and, though the results may be expressed very briefly, it is thought that their importance warrants their publication as a supplementary note to the paper referred to above.

Two hundred specimens of normal urine were available, each obtained from a healthy individual; in all cases the urine was fresh, and had not stood long after being passed. The method of estimating the hæmolytic activity was the same as that adopted in the paper mentioned above, to which the reader is referred for all details. The titre of a urine was taken as the smallest amount which could completely hæmolyse 1 c.c. of the standard blood suspension, when the mixture of blood and urine was incubated at 37° C. for four hours. Readings of results were taken after this period, and also at a period 2½ hours after incubation was begun.

The following were the results obtained:

(1) Of the 200 urines examined 19 per cent. produced hæmolysis of the suspension in 2½ hours, while 64 per cent. produced hæmolysis in 4 hours. These figures bear out the observation previously recorded, that the number of positive results obtained after 2½ hours' incubation is about one-third of the true number of hæmolytic specimens.

(2) Of the 128 specimens which proved to be hæmolytic 28 per cent. had a hæmolytic titre of 0·1 c.c. or less, 12 per cent. had a titre of 0·2 c.c., 14 per cent. a titre of 0·3 c.c., while the remaining specimens had a titre of 0·4 c.c. Specimens, 0·4 c.c. of which did not produce hæmolysis, were considered non-hæmolytic.

(3) Thirty-six per cent. of the 200 specimens were non-hæmolytic.

Several points may be noted:

(1) Since the hæmolytic activity of the urine excreted from day to day

varies, at times there being no hæmolytic power detectable, while at other times great activity is displayed, we may safely assume that had the 72 negative specimens been examined on subsequent days, the majority would have shown a hæmolytic activity. This, unfortunately, was not practicable.

(2) The difference between these results and those published in the previous paper is intelligible when it is remembered that the number of cases then examined was not so large, and that, though the cases were normal, they were selected because of the occurrence of hæmolytic substances; the estimation of the hæmolytic activity on many consecutive days in each of these cases would tend to give a higher percentage of positives than would be found in unselected normals.

(3) Since the percentage number of positives obtained by the examination of these 200 normals is distinctly less than the percentage number obtained from 300 cases of insanity, in which one estimation only was made (see paper referred to above), and since both these percentages are based on the examination of numbers of cases large enough for the results to be considered representative, it seems probable that there is a greater tendency to the excretion of hæmolytic substances in the urine among the insane, and doubtless also in many other pathological conditions, than there is in the normal individual. It is obvious, on the other hand, that the occurrence of such substances is not in itself abnormal, nor is it necessarily the accompaniment of a pathological condition.

CONCLUSIONS.

Sixty-four per cent. of normal unselected urines were found to be hæmolytic. If the non-hæmolytic specimens had been examined repeatedly they would probably have been found to be hæmolytic at some time. It seems that the presence of hæmolytic action is more frequent in insanity, but in itself is not abnormal.

REFERENCE.

1. Ponder, E.—"The Presence of Hæmolytic Substances in Human Urine," *Brit. J. Exper. Pathol.*, 1921, **2**, 34.

THE PHYSICO-CHEMICAL STATE OF SUGAR IN THE BLOOD.

KANTARO ONOHARA.

From the Second Medical Clinic, Medical College, Osaka.

Received for publication March 30th, 1921.

In spite of many and varied attempts, no decisive answer has yet been given to the question whether the glucose of the blood is present in the free crystalloid state, or whether it exists in the form of some colloidal complex. Henriques and Bing consider that most of it is in combination with lecithin as a complex resembling or identical with Drechsel's jecorin, and Kolisch, from clinical observations, holds the same view. Otto Loewi's experiments on pancreatic and phloridzin glycosurias led him to the similar conclusion that glucose was present normally in the form of a colloid complex, but that in the hyperglycæmia of pancreatic diabetes the excess was present in the crystalloid state, and thus was excreted at an increased rate when diuretics were given, just as urea and salts were. Lusk also believed that there were sugar-colloid combinations in normal blood.

Schenk, and later Rosenfeld, on the other hand, having demonstrated that the dialysability of glucose was not altered by the presence of protein or blood, regarded it as being present in the free state, though these conclusions have been criticised on theoretical grounds by Asher. Meyer contended that the free dialysability of the sugar was not incompatible with its normal existence in the blood in colloid form, unless the absence of enzymes capable of decomposing jecorin could be ensured. His search for such enzymes was, however, fruitless. Pflüger's arguments against the presence of free glucose appear to have been equally inconclusive, and the experiments of Michaelis and Rona, which showed some promise, were discountenanced by their later work.

It seems desirable that the problem, being of a physico-chemical nature, should be attacked by physico-chemical methods. At the suggestion of Prof. Kozawa use was made of an observation made by him in 1914.(1) He found that glucose can diffuse into human red corpuscles, with the result that their volume, as judged by the hæmatocrite volume of the blood, is increased. For this reason, as he showed in 1919(2), the red cells in diabetes mellitus are remarkably larger than the normal.

TECHNIQUE.

Osmotic experiments were made after the method described by Kozawa, which is briefly as follows:

He employed as the osmotic membrane human red corpuscles, which were mixed with the various experimental fluids, isotonic glucose solution (5·6 per cent.), normal serum, or the serum of a glycosuric patient having an excess of sugar. The volume of the red corpuscles thus treated was measured by the hematocrite. The sugar content was estimated by MacLean's modification of Bertrand's method.(3)

RESULTS.

Results with Human Red Corpuscles.

(1) The results of hematocrite examinations carried out by mixing equal parts of human red corpuscles (washed several times with sterilised physiological saline) and normal human serum by centrifugalising in a highly efficacious electric centrifuge, showed that there was no difference in the size of the red corpuscles. (The figures in the following tables are the hematocrite indices.)

TABLE I.

Sugar content.	Immediately.	1 hour.	2 hours.
0·102 per cent.	37	37	37
0·098 ,,	37	37	37

(2) My experiments also prove that glucose penetrates into human red corpuscles in isotonic glucose solution and increases the bulk of the cells, as is shown in the following table:

TABLE II.

Immediately.	½ hour.	1 hour.	1½ hours.	2 hours.	3 hours.	24 hours.
46	48·5	49	52·5	53	53·5	57

(3) The results of the experiments made with an isotonic saline-glucose solution and normal human red corpuscles were as follows, being similar to those of Table II:

TABLE III.

Sugar contents.	Immediately.	1 hour.	2 hours.	3 hours.	24 hours.
0·100 per cent.	41	41	41	41	—
0·280 ,,	42	42	42	42	—
1·000 ,,	43	43·5	43·5	44	44
2·000 ,,	47	48	48	49	49
2·800 ,,	48	54	58	59	—

(4) The hematocrite indices of normal red corpuscles treated with normal human serum with the addition of isotonic glucose solution are shown in the following table. It will be seen that there is no difference in the diffusion whether the solvent be serum or isotonic saline glucose solution.

TABLE IV.

Sugar contents.	Immediately.	1 hour.	2 hours.	3 hours.	24 hours.
0·776 per cent.	43	43	43	—	—
1·469 ,,	49	52	52	54	54

(5) The hematocrite indices of normal human red corpuscles in the serum of the diabetic patient (having an excess of sugar content) were as follows: They are practically the same as those obtained by the use of either isotonic saline-glucose solution or serum with the addition of isotonic glucose solution. By this evidence it is clear that there is no difference between the behaviour of sugar in the blood and that of free sugar as regards diffusion into the red corpuscle. In other words it may be concluded that sugar in the circulating blood shows the same properties as regards diffusion through the plasma-membrane of the human red corpuscles as free glucose does.

TABLE V.

Sugar contents.	Immediately.	1 hour.	2 hours.	3 hours.	24 hours.
0·776 per cent.	43	43	43	—	—
1·035 ,,	48	49	51	53	53

CONCLUSION.

(1) As shown by Kozawa, glucose causes swelling of human red corpuscles But practically the same hematocrite results were obtained with human red corpuscles treated with the serum of a diabetic patient, normal serum with the addition of isotonic glucose solution, or isotonic glucose-saline solution.

(2) It may therefore be assumed that sugar in the circulating blood is present in the same physico-chemical character as in the solution of free glucose. In other words, it must be present in the circulating blood in the form of free sugar.

I wish to express my sincere thanks to Prof. Kozawa, who suggested this work to me and helped me throughout the course of the experiments.

REFERENCES.

1. KOZAWA, S.—"Beiträge zum arteigenen Verhalten der roten Blutkörperchen," *Biochem. Zeitschr.*, 1914, **60**, 231.
2. *Idem.*—"Note on the Permeability of Red Corpuscles for Glucoses and Glucosamine," *J. Physiol.*, 1919–20, **53**, 264.
3. GARDNER, A. D., AND MACLEAN, H.—"A Method for the Estimation of Sugar in Blood, with Observations on some Modern Methods," *Bio-Chem. J.*, 1914–15, **8**, 391.

GROUPING OF INFLUENZA BACILLI.

S. YABE,

Kitasato Institute for Infectious Diseases, Tokyo.

Received for publication June 1st, 1921.

EITHER for preparing vaccine or for immunisation, the first requisite is to select suitable strains of influenza bacilli. I began, therefore, at the commencement of my experimental immunisation, to study to what extent strains varied as soon as it was concluded that the last influenza pandemic was caused by Pfeiffer's bacilli. The results of my work, with reference to vaccine and immune serum(1) were published in Japan in March, 1919. As reports on the varieties of influenza bacilli are now under discussion, a part of my experimental study dealing with various strains may be summarised here again. It is of interest not only as a problem in immunity but also in connection with the ætiology of the influenza pandemic.

Even at the time of the discovery of the influenza bacillus a suspicious strain was isolated from a child with broncho-pneumonia following diphtheria by Pfeiffer (2) himself, which he, being unable to differentiate distinctly from the typical strains by morphology, designated *Bacillus pseudo-influenza*. From time to time the "pseudo-influenza bacilli" have been isolated by various authors both from healthy or unhealthy respiratory tracts. Some were very similar in form to the typical influenza bacilli, but were non-pathogenic. Thus the very interesting question as to which was the real pathogenic germ of epidemic influenza remained unsettled up to the time of the last pandemic.

Before this last pandemic Grassberger (3) and Wollstein (4) reported varieties in the form of influenza bacilli, the last also showing immunity differences between different strains. More recently, independently of my study, Edwin O. Jordan (5) reported that ten strains produced indol while three did not. So far as differences in immunity reactions are concerned, Parker (6), Valentine and Cooper (7), Fleming and Clemenger (8), Small and Dixon (9) and Howard H. Bell (10) studied agglutination with a certain number of strains and their respective immune sera for different purposes. Some of them intended from the first to divide the strains into groups. No one, however, could divide the strains into certain definite groups, but each saw great differences among the strains. J. V. Cooke (11), who tried complement-fixation with sixteen strains, detected "no definite antigenetic relationship."

From my experience the morphological difference is not fixed enough to divide the strains into groups. Agglutination also yields no reliable results with influenza bacilli, as they are liable to spontaneous agglutination. Phagocytosis is too sensitive. Hence among the immune reactions complement-fixation seems most stable, even though the antigens are apt to be easily destroyed as shown below.

THE DIFFERENCE IN INDOL FORMATION.

The absolute distinction between strains of influenza bacilli is in the property of indol formation. Jordan was the first to show the production of indol by certain strains. He reported in June, 1919, that in his specially designed culture medium ten out of thirteen strains produced indol. In January, 1919, when it was important that certain strains should be chosen for the purpose of making vaccine and immune serum, I obtained proof of indol formation in the following way: The ordinary slightly alkaline bouillon was heated over 90° C. in a Koch's steriliser, taken out, and defibrinated horse blood added in a 10 per cent. proportion. It was then thoroughly shaken to dissolve the blood-corpuscles. When it changed to a dark brown mixture it was filtered, first through filter-paper and then through Berkefeld's clay-filter. The final filtrate was collected in a sterilised flask and then divided into sterilised test-tubes, 10 c.c. for each. To test its sterility it was kept at 37° C. for a day. If sterile, it remains quite clear, tinged a little yellow. A platinum loop of culture of influenza bacilli was then inoculated into it. The strains inoculated were isolated from the expectoration of typical influenza cases, simple or complicated. The indol reaction was tested after the Kitasato-Salkowsky method.

TABLE I.—*Indol Formation by New and Old Strains in Four Days' Growth.*

The new strains had been isolated for two months, and the old strains had been isolated fifteen to eighteen months before: *n* for the new, *o* for the old strains.

Number of strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
New or old	n	n	n	n	n	n	o	o	o	o	o	o	o	o	o
Indol in four days' growth	−	+	+	+	+	−	+	+	+	−	−	+	−	−	+

The table shows two negative strains out of six new strains, and four negative ones out of nine old. It seems most probable that subculture (made every fourth day) has no influence upon the property of indol formation.

TABLE II.—*Indol Formation by several Strains in Twelve Days' Growth.*

Number of strain	16	17	18	19	20	21	22	23	24	25	26	27	28	29	1	7
Indol in twelve days' growth	+	+	−	+	+	−	−	+	+	−	+	−	+	+	−	+

Among 29 old and new strains tested there are 18 positive indol strains (62·0 per cent. of the total) and 11 negative indol (38·0 per cent. of the total number of strains). Some strains seemed to produce indol a little quicker than others, owing probably to the rapidity of their growth, but in full growth there was no noticeable difference. When I referred to patients' records no relation was found between the production of indol and the severity of disease.

Hence the influenza bacilli were definitely differentiated into two characteristic groups—one positive indol, the other negative indol.

THE DIFFERENCE IN THE VIRULENCE AND TOXICITY OF THE TWO GROUPS.

Wollstein reported a difference between the virulence of a strain from blood in a case of meningitis and of another from the respiratory tract in bronchitis. The virulence was strong in the strain from meningitis—a 1 kgrm. rabbit being killed in 18 to 30 hours after the inoculation of an agar slant culture, while that from the respiratory tract was avirulent. In examining the virulence of influenza bacilli the length of time they have been maintained in artificial culture should be taken into account. In my strains the newly isolated cultures from typical influenza had usually a strong virulence, killing in a day a 10 grm. mouse with 0·1 to 0·2 mgrm. culture, or a 200 grm. guinea-pig with 1·0 to 2·0 mgrm. when injected into the peritoneal cavity. On the other hand, the virulence of strains from carriers proved very weak, but strengthened with the onset of the fever. For instance, a new culture, isolated in November, 1918, from my laboratory girl when she was a carrier, did not kill a 10 grm. mouse with even 2 mgrm., while the first culture when isolated on January 4th, 1919, during the attack of fever killed a mouse of the same weight with 0·05 mgrm.

All strains, however, lost a great deal of their original virulence with each subculture and finally became avirulent. As the decrease of virulence on subculture is not the same with each strain, it is unreasonable to compare the virulence of some strains with that of others, either in the same or in different generations of the culture, except in the first generation. Summed up, there was no remarkable difference of virulence between the positive indol strains and the negative, as determined by the minimal lethal dose of the first culture.

In order to determine the toxicity sixteen strains were tested, eight strains being positive indol and the others negative. The eighteen hours' growth of each strain on heated blood-agar was emulsified separately strain by strain in 0·5 per cent. carbolic saline at the rate of 1·0 mgrm. in 1·0 c.c. After standing the emulsions for two hours in the incubator at 37° C. separate strains were injected into rabbits intravenously. It was proved by cultivating that the bacilli in the emulsion were killed at the time of injection, hence there was no time for the toxicity to diminish by the decomposition of the killed bacilli. The quantity injected was 1 c.c. containing 1 mgrm., or when diluted 0·5 mgrm. killed bacilli. The injected rabbits which died were weighed and an autopsy made occasionally. They were observed for ten days after the injection. Table III gives the result of this experiment.

The susceptibility of rabbits to the bacillary toxin is somewhat variable, but it seems that the toxicity is a little stronger in negative indol strains than in positive. The weight of the animals is very much diminished in the case

TABLE III.—*The Toxicity of Strains.*

Rabbits.	Weight before injection.	Number of strain.	Indol formation.	Quantity injected		Survived or succumbed.	Post-mortem examination.	
				Actual	Per kgrm.		Weight.	Special changes.
I	2355 grm.	24	+	1 c.c. = 1·0 mgrm.	0·42 mgrm.	†3*	2115 grm.	General anæmia, spotted bleeding in lungs.
II	1970 ,,	4	+	Ditto.	0·50 ,,	†4	1800 ,,	General anæmia, spotted bleeding in lungs, diarrhœa.
III	2090 ,,	28	+	,,	0·49 ,,	—	—	—
IV	2235 ,,	7	+	,,	0·44 ,,	—	—	—
V	2175 ,,	23	+	,,	0·46 ,,	—	—	—
VI	2920 ,,	12	+	1 c.c. = 0·5 mgrm.	0·17 ,,	†1	—	—
VII	2175 ,,	15	+	Ditto.	0·23 ,,	—	—	—
VIII	2750 ,,	9	+	,,	0·18 ,,	—	—	—
IX	2950 ,,	12	+	,,	0·17 ,,	—	—	—
X	2380 ,,	27	—	1 c.c. = 1·0 mgrm.	0·42 ,,	—	—	—
XI	2755 ,,	25	—	Ditto.	0·36 ,,	†5	1945 grm.	General anæmia, bleeding in lungs and pericardium.
XII	2175 ,,	22	—	,,	0·46 ,,	†1	1905 ,,	Hyperæmia in colon, diarrhœa, spotted bleeding in lungs.
XIII	2650 ,,	11	—	,,	0·38 ,,	†1	2630 ,,	Bleeding in lungs, ovary and supra-renal glands.
XIV	1975 ,,	10	—	,,	0·50 ,,	†3	1530 ,,	Hyperæmia and bleeding in lungs.
XV	2500 ,,	1	—	1 c.c. = 0·5 mgrm.	0·20 ,,	—	—	—
XVI	2620 ,,	6	—	Ditto.	0·20 ,,	—	—	—
XVII	2220 ,,	13	—	,,	0·23 ,,	—	—	—

* †1 or †2 means death one day or two days after the injection.

of death a few days after the injection. Diarrhœa usually occurs in acute intoxications. The animals which survived the injection for a few days had marked hæmorrhages.

CROSS COMPLEMENT-FIXATION WITH ANTIGENS AND IMMUNE SERA OF SEVERAL STRAINS OF THE TWO GROUPS.

According to my experiments complement-fixation seems to be the most stable and reliable immune reaction. Agglutination is not reliable as the bacilli are so liable to undergo spontaneous agglutination, and it was found impossible to prevent this. Complement-fixation was therefore chosen both for the standardisation of immune serum or for the classification of influenza bacilli.

Six strains were used to obtain their respective immune sera, three being positive indol and the other three negative indol. Rabbits were intravenously injected separately, strain by strain. Each was twice injected with 0·5 mgrm. bacilli killed by carbolic acid, and at the period of the highest immunity they were bled. No disinfectant for conservation was added to the sera which were tested a few days after. Before testing each serum was heated at 57° C. for half an hour.

Antigen was made as follows: The 48 hours' growth of each strain on heated blood agar was separately emulsified in 0·5 per cent. carbolic-saline in the proportion of 2 mgrm. in 1 c.c. fluid. This emulsion was kept at 37° C. overnight so that the bacilli were killed and moderately autolysed. It is most important that there should be a certain degree of autolysis in the antigen that it may be effective. The antigen is killed in an incubator rather than at a higher temperature, because too much autolysis may occur and the antigen become ineffective.

The hæmolytic system was sheep's red corpuscles (5 per cent. suspension of blood-corpuscles) with a rabbit *versus* sheep's immune body.

As complement guinea-pig's fresh serum was used. For use it was preliminarily tested, so that the quantity employed was 0·5 c.c. diluted fourteen times—that is, double units for this system. Next the antigens were tested for their anti-complementary effect in this system and also for their antigenic power with regard to the antibody.

TABLE IV.—*The Anti-complementary Effect of each Antigen.*

Each tube contains 0·5 c.c. antigen; its corresponding weight of bacilli is shown in figures: H = complete hæmolysis; **D** = complete deviation; D = moderate deviation; d = slight deviation.

Number of strain.	Quantity of antigen in each strain (mgrm.).			
	0·5	0·25	0·12	0·06
12	H	H	H	H
13	H	H	H	H
1	d	H	H	H
15	H	H	H	H
6	**D**	d	H	H

TABLE VI.—*Showing the Antigenic Effect of* 0·12 *mgrm. of each Strain of Influenza Bacilli on its own as well as on Immune Sera of the Other Strains, cross-examined as shown. The immune serum is diluted so that each tube contains 0·5 c.c. To the first tube of the first antigen tested in each series saline was added instead of antigen as a control of the anti-complementary effect of the serum.*

The quantity of serum in the columns numbered 1 to 6 are: No. 1, 0·05 c.c.; No. 2, 0·025 c.c.; No. 3, 0·012 c.c.; No. 4, 0·006 c.c.; No. 5, 0·003 c.c.; No. 6, 0·0015 c.c.

With the immune serum of No. 12 (positive indol strain).

Indol.	Antigen. No.	Quantity of the immune serum.					
		1	2	3	4	5	6
–	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	D	H
–	1	**D**	**D**	**D**	D	H	H
–	13	**D**	**D**	**D**	**D**	D	d
+	12	D	H	H	H	H	H
+	9	**D**	**D**	**D**	D	d	H

With the immune serum of No. 6 (negative indol strain).

–	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	**D**	d
–	1	**D**	**D**	**D**	**D**	D	H
–	13	**D**	**D**	**D**	**D**	d	H
+	12	**D**	D	d	H	H	H
+	9	**D**	**D**	D	d	H	H

With the immune serum of No. 13 (negative indol strain).

–	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	D	H	H
–	1	**D**	**D**	**D**	D	H	H
–	13	**D**	**D**	**D**	D	H	H
+	12	H	H	H	H	H	H
+	9	d	H	H	H	H	H

With the immune serum of No. 1 (negative indol strain).

Indol.	Antigen No.	Quantity of the immune serum.					
		1	2	3	4	5	6
–	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	d	H
–	1	**D**	**D**	**D**	**D**	**D**	**D**
–	13	**D**	**D**	**D**	**D**	D	H
+	12	**D**	D	H	H	H	H
+	9	**D**	D	H	H	H	H

With the immune serum of No. 15 (positive indol strain).

–	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	D	H
–	1	**D**	**D**	**D**	D	H	H

With the immune serum of "H" strain (indol formation was not examined).

–	6	H	**D**	**D**	**D**	**D**	**D**
+	15	**D**	**D**	**D**	**D**	**D**	**D**
–	1	**D**	**D**	**D**	D	d	H
–	13	**D**	**D**	**D**	**D**	**D**	D
+	12	**D**	**D**	D	d	H	H
+	9	**D**	**D**	**D**	**D**	d	H

TABLE V.—*Showing the Antigenic Effect on the Serum of a Rabbit which was Strongly Immunised with Strain No. 12.*

The volume of antigen in each tube is 0·5 c.c.; the weight of bacilli contained corresponds to the figures shown. To each tube 0·5 c.c. of the immune serum six times diluted was added.

Number of strain.	Quantity of antigen in each strain (mgrm.).					
	0·5	0·25	0·12	0·06	0·03	0·015
12	**D**	**D**	**D**	d	H	H
13	**D**	**D**	**D**	**D**	d	H
9	**D**	**D**	**D**	**D**	d	H
1	**D**	**D**	**D**	d	H	H
15	**D**	**D**	**D**	D	d	H
6	**D**	**D**	**D**	**D**	D	d

From these two preliminary tests 0·12 mgrm. of each antigen was found to be the optimum amount—that is, 0·5 c.c. four times diluted—and this quantity was then cross-tested with various immune sera as shown in Table VI.

As the table shows, no definitive antigenic relationship could be detected between the positive and negative indol groups, nor among any of the strains examined. Nevertheless some yield a much more potent antigen than others even when prepared in the same way, and this may account for the fact that a particular antigen does not necessarily react most strongly with its respective immune serum. Thus the varying effect of each antigen may be due mainly to the properties of each strain. Strains No. 6 and No. 9 were very liable to yield to spontaneous agglutination and seemed to be closely allied. Their antigenic effects, however, were quite different, the former being very strong, the latter weak.

CONCLUSIONS.

(1) Influenza bacilli can be divided definitely into two groups according to the character of indol formation. Out of 29 strains examined, 18 strains (62 per cent.) form indol. No intermediate strains are found. It is very probable that this character of indol formation is not alterable by prolonged subculture.

(2) By morphological and immunological study it is impossible to divide influenza bacilli into groups, because there are found as many intermediate strains as there are strains examined.

REFERENCES.

1. YABE, S.—*Tokyo Yijishinshi*, 1919, No. 2118, p. 1.
2. PFEIFFER, R.—"Die Ætiologie der Influenza," *Zeitschr. f. Hyg. u. Infektionskrankh.*, 1893, **13**, 357.
3. GRASSBERGER, R.—"Beiträge zur Bakteriologie der Influenza," *ibid.*, 1897, **25**, 453.
4. WOLLSTEIN, M.—"An Immunological Study of Bacillus influenzæ," *J. Exper. Med.*, 1915, **22**, 445.

5. JORDAN, E. O.—"The Production of Indol by Certain Strains of the Pfeiffer Bacillus," *J. Amer. Med. Assoc.*, 1919, **72**, 1542.
6. PARKER, J. T.—"The Poisons of the Influenza Bacillus," *J. Immunol.*, 1919, **4**, 331.
7. VALENTINE, E., AND COOPER, G. M.—"On the Existence of a Multiplicity of Races of B. influenzæ as Determined by Agglutination and Agglutinin Absorption," *ibid.*, 1919, **4**, 359.
8. FLEMING, A., AND CLEMENGER, F. J.—"The Specificity of the Agglutinins produced by Pfeiffer's Bacillus," *Lancet*, 1919, **2**, 869.
9. SMALL, J. C., AND DICKSON, G. K.—"Grouping of Bacillus influenzæ by Specific Agglutination," *J. Infect. Dis.*, 1920, **26**, 230.
10. BELL, H. H.—"Relation of Different Strains of Influenza Bacilli as shown by Cross-agglutination and Absorption Tests," *ibid.*, 1920, **27**, 464.
11. COOKE, J. V.—"Complement-fixation in Influenza with B. Influenzæ Antigens," *ibid.*, 1920, **27**, 476.

THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

CAPILLARY BLOOD-PRESSURE AND THE GLOMERULAR FILTRATION THEORY.

LEONARD HILL AND JAMES McQUEEN,

National Institute for Medical Research, London.

Received for publication June 17th, 1921.

ACCORDING to Cushny,(1) "The function of the kidney may be shortly defined as the filtration of the non-colloid constituents through the capsule and the absorption of 'Locke's fluid' through the tubule cells. The capsule furnishes the tubules with the fluid as it exists in the circulation, the tubules return to the blood the fluid best adapted for the tissues, and allow the rest to escape in the urine."

The modern theory of the secretion of the urine, as enunciated by this author, stands or falls by an accurate measurement of the blood-pressure in the capillaries of the glomeruli. The theory postulates that protein-free plasma is filtered through each glomerulus without expenditure of energy by the cells which encapsulate it, the energy of filtration being derived from the action of the heart. Reabsorption of this fluid occurs through the vital activity of the renal tubule cells.

According to the modern theory, which assumes that the glomerulus is comparable to a gelatine filter, the possibility of filtration through it depends on (*a*) the actual capillary pressure available to separate the water, sugar and salts from the blood; (*b*) the osmotic pressure of the blood colloids, which may be put at about 25–30 mm. Hg., and which will be less according as the blood colloid is diluted.

Now, there are two well-established facts in the physiology of the kidney with which this filtration theory of the glomerular function must come into line. To take the first, the maximum to which the secretory pressure rises in the ureter and pelvis of the kidney lies between 60–70 mm. Hg. It may be lower; under conditions of saline diuresis it may be higher. But taking the figure of 60 mm. Hg. pressure, the modern view of kidney function recognises that the capillary pressure in the glomerulus must, when the ureter pressure is 60 mm. Hg., reach a pressure of 90 mm. Hg., because the capillary pressure must over-top the ureter pressure by 30 mm. Hg., if the osmotic pull of the proteins on the water of the blood is to be overcome and filtration ensue. Accordingly the modern view, while admitting that this estimate of the glomerular capillary pressure is at the best mere guesswork, states that the capillary pressure may be taken as 20 per cent. below carotid pressure.

The assumption of a pressure 20 per cent. less than the aortic or carotid pressure is necessitated by a consideration of the second well-established fact in the physiology of the kidney. The minimum pressure in the aorta or carotid below which urine fails to be secreted from normal undiluted blood is about 40 mm. Hg. Deducting from the 40 mm. Hg. 20 per cent. of its value, a glomerular capillary pressure is left of 32 mm. Hg.—an amount capable of overcoming the osmotic pull of the blood colloids on the water and maintaining filtration.

Now let us consider what this assumption involves. If the carotid blood-pressure is 120 mm. Hg. the glomerular capillary pressure is 20 per cent. less, or 96 mm. Hg. If the aortic blood-pressure is 40 mm. Hg. the glomerular capillary blood-pressure is 20 per cent. less, or 32 mm. Hg. The loss of pressure in both cases is due to friction of the blood passing from arteries through arterioles to capillaries. The pressure gradient indicates the loss due to friction. Now, while the kidney is actively secreting it swells owing to vaso-dilation; even when the blood-pressure is very low, viz. 40 mm. Hg., the renal arterioles may be, and most probably are in a state of vaso-dilation. While the pressure gradient in the first instance is assumed to be 24 mm. Hg., in the second instance a pressure gradient of 8 mm. Hg. is postulated. It does not seem likely that the pressure gradient in the same vessels, and over the same course, should vary in this manner.

The consideration of these figures shows that the 20 per cent. reduction in aortic pressure is a figure arbitrarily chosen to suit the filtration theory of glomerular function. If the gradient were in both cases 24 mm. Hg., when the aortic or carotid blood-pressure is 40 mm. Hg., there would remain only 16 mm. Hg. available to separate blood colloid from the water, and filtration could not occur at that pressure as long as the colloidal pull reached its normal figure of 20–30 mm. Hg. The value of the gradient has to be sacrificed to maintain consistency in fundamental portions of the filtration theory.

In regard to the problem of the pressure values in the arteries, arterioles and capillaries of the mammalian kidney, only indirect proof is at present available as a basis of argument. But there are certain measurements that can be and have been accurately obtained. The pressure in the renal veins of a dog was found by Burton Opitz (2) to be 10·9 mm. Hg. We know from the work of L. Hill (3) on animals that a very low pressure pertains in the capillaries not only of the frog, but in the ear and abdominal muscular wall of the mouse and in the wing of the bat, and Hill and McQueen (4) have measured the arteriole pressure in the skin of the hand and this is roughly 10 mm. Hg. at heart level—one-third to one-fifth of almost all previous estimates. Basler (5) found the pressure of blood, flowing from a cut in the finger, raised the mercury in a manometer 7–9 mm. In cutting the finger arterioles will be wounded, and this may be taken then to be the arteriole pressure. The pressure in the veins leading from the capillary areas of the hand in man is 0·7–6·7 mm. Hg., (6) that is a few millimetres below the arteriole pressure—sufficient to ensure a pressure gradient. Consequently it is reasonable to suppose that, with a renal vein pressure of 10·9 mm. Hg. the pressure in the second capillary network of the kidney lies in the neighbourhood of 12–13 mm. Hg.

It is possible to arrive at a fairly accurate estimate of the pressure in the small arteries of the kidney substance, for if the ureter is obstructed, we know that up to 40 mm. Hg. pressure in the ureter (no less pressure must pertain in the renal tubules and capsules) no interference with the blood-flow from the renal vein occurs (Cushny). At 50 mm. Hg. pressure Lindman found the venous outflow was impaired. Confined as the kidney is by its capsule, all the smaller arteries, including the afferent arteries of the glomeruli, the capillaries and venules, are compressed by this 50 mm. Hg. pressure which pertains from the pelvis through the tubules, less such part, if any, of the pressure as the *membranæ propriæ* of the tubules and capsules shield the vessels from. These membranes in the kidney do not withhold pressure to the same extent as in the salivary gland, where the secretory pressure can rise above aortic systolic pressure without arresting the circulation through the salivary gland. Consequently the blood-pressure in the venules and capillaries rises towards this 50 mm. Hg. pressure until the smaller arteries are diminished in calibre, and this occurs when the diastolic pressure in these arteries is overcome. Hence diminution of venous outflow means the overcoming of the diastolic pressure in the small arteries. We constructed a scheme in which this diminution of venous outflow was demonstrable on overcoming the diastolic pressure in the artery.(7) Now we know that the range between diastolic pressure and systolic pressure is limited in small arteries. Hence the systolic pressure in the small arteries of the kidney is probably not higher that 60 mm. Hg.

A pressure range of 50–60 mm. Hg. in the small arteries of the kidney is not very different from the pressure in the small arteries of the hand, or in the main artery of the proximal segment of the bat's wing, which has been directly measured by one of us, hence we are able to estimate the probable fall of pressure between these arteries and the capillary field of the glomerulus.

It has been generally held that the pressure of the two capillary fields athwart the blood path in the kidney requires of necessity a high blood-pressure to maintain circulation. Barcroft and Straub have shown that with large injections of saline a secretion of urine has taken place with no more than 12 mm. Hg. pressure in the aorta. Of course in this case the colloid of the blood was diluted and viscosity diminished, but it would appear that the long path of the double capillary field can be traversed by fluid at very low pressure. In this case the 20 per cent. reduction would reduce the glomerular capillary pressure to 10 mm. Hg. When the excised kidney is perfused at 15–20 mm. Hg. pressure, fluid escapes from the ureter in some quantity, and the perfusion fluid circulates through the two capillary fields. All this is in accordance with the findings of L. Hill, viz. that a pressure of 1–2 mm. Hg. suffices to make a flow in the capillaries of the web of the amputated leg of the frog. The theory of great resistance in the capillary bed is erroneous. The pressure gradient does not fall much in capillary fields, but during the passage of blood through the smaller arteries and arterioles. L. Hill has found in the bat's wing that the pressure in arterioles branching at a right angle (the afferent artery to each glomerulus is such) falls to about one-half of the pressure in the main stems, *e.g.* from 50 to 20–25 mm. Hg. In the case of a schema with a narrow tube opening into a wider one and this in its turn into a narrow one, the lateral pressure in the wider part actually exceeds that in the entrance tube. Such a schema cannot be justly used to interpret the pressures in the vessels of the glomerulus, because the arteriole of the glomerulus divides into a large number of capillaries and these reunite into the afferent vessel, so that, as can be demonstrated in a schema so constructed, lower pressures exist in each individual capillary than pertain in the arteriole. Accordingly all the available data tend to show that if the small artery pressure be 50–60 mm. Hg., the pressure in the capillary field of the glomerulus falls as in other capillary fields which are accessible to direct measurement. Consequently the capillary field in the glomerulus has a blood-pressure at most some few millimetres higher than in the second capillary field. We have seen that, according to the modern theory of renal secretion, a pressure of 96 mm. Hg. is assumed for the glomerular capillaries when the aortic pressure is 120 mm. Hg. and we have reached the conclusion that the second capillary field in the kidney has a pressure a few millimetres above the renal vein pressure. It would be truly remarkable if, glomerular pressure being assumed by the theory to be 20 per cent. less than aortic, 24 mm. Hg. pressure were lost in passing from the aorta to the capillaries of the glomerulus, and 85 mm. Hg. pressure were lost in passing from the glomerulus through the second capillary field to the renal vein.

We now turn to another point. The modern theory supposes that a glomerular capillary pressure of some 90 mm. Hg. filters protein-free plasma through Bowman's capsule till the pressure in the obstructed ureter reaches 60 mm. Hg., when the filtration ceases, because some 30 mm. Hg. pressure is required to separate the water from the colloids of the blood. No attention appears to have been paid by those upholding this theory to the general condition of the kidney in this experiment. Cushny says that the pressure in the ureter is propagated along the tubules to the capsule, and this opposes the filtration pressure. It must equally be propagated through the walls of the tubules to the surrounding capillaries and venules. It will be found that the kidney becomes tense and hard, which is proof that every structure in it is under pressure, being confined within the resisting structure of the kidney capsule. Supposing the pressure in the glomerular capillaries rises to the maximum pressure in the ureter, viz. 60 mm. Hg., this can only result from the compression of venous exits by the pressure of the urine in the renal tubules and capsules. The high capillary pressure so induced is artificial, and does not indicate the normal conditions.

We may here recall an experiment of Henderson (8). He found the pressure in the ureter fell on inhibiting the heart, and ascribed this to retraction of the glomeruli and fluid in the tubules escaping into the capsules so enlarged. Against this view must be put the valvular action of the openings of the collecting tubules into the pelvis, which, as Cushny has shown, prevent any escape of fluid from the pelvis even when the kidney substance is cut away almost till the pelvis is reached. The true explanation is that the pelvis becomes distended when the ureter is obstructed, and presses in upon the kidney, which resists compression owing to the high vascular pressure pertaining in arteries, capillaries and veins confined by the renal capsule. When the heart is inhibited the kidney's resistance to deformation is lessened, the pelvis swells, its capacity increasing, and so the pressure within it falls, as has been observed by J. McQueen and A. Webster.

We may also recall the interesting experiment of Alcock and Lewi of enclosing the kidney in plaster-of-Paris so that it cannot expand. Injection of saline caused diuresis, and the renal venous blood became brighter in colour. The conditions here are comparable to the brain or eye or salivary gland enclosed in a capsule strictly limiting expansion of the organ. Dilatation of arteries brings about constriction of veins, and the whole system—arteries, capillaries and veins—approximates to a rigid system with rapid blood-flow, which in its turn favours diuresis.

The important point to note is that when compression is applied over the whole organ the blood-flow is diminished so soon as the diastolic pressure in the small arteries is overcome. The compressed arteries then open intermittently with each beat of the pulse, and blood flows through them only so long as the level of systolic pressure overtops the compressing pressure, as has been observed in the frog's web, bat's wing, etc., by L. Hill. For this reason secretion ceases before systolic pressure can be attained in the ureter.

The pressure gradient deduced in the kidney when actively secreting and in a state of vaso-dilatation, according to the above argument, may then be stated thus:

Small arteries	50–60	mm. Hg.
Glomerular capillaries . .	15–20	,,
Second relay of capillaries .	13–15	,,
Renal vein	11	,,

There remains for discussion certain corroborative evidence of the accuracy of these findings.

We are fortunate in having for consideration well-authenticated results due to various workers drawn from experiments on the action of saline injections into animals. Any consideration of the influence of the action of saline injections in inducing dilution of the blood colloids and promoting diuresis must carry with it the consideration of the influence of colloid dilution by salines on the capillaries of the body generally. When saline solutions are injected into animals, water and salts pass not only through the glomerular capillaries but through those of the body generally (Magnus). Saline injections permeate equally well through capillary systems where the capillary blood-pressure has been shown to be less than 10 mm. Hg. Dilution of the colloids of the blood has then a precisely similar effect where the capillary pressure is known to be low as it has in the case of the renal capillaries, *e. g.* of the glomerulus, wherein the blood-pressure, according to the modern view, is believed to be 20 per cent. below the aortic pressure. The sole difference in the two cases is that the water and the salts escape through the ureter in the one case, and escape through the lymph into the blood-stream or are retained in the tissues in the other case. Evidently the physiological permeation of salts or water through capillaries can take place with the very low capillary pressure which has been determined by L. Hill. In place of filtration it might be argued that the water and salts pass through the capillary in virtue of the blood having a lower osmotic pressure than the tissue cell. With normal blood a colloidal osmotic pull of 20–30 mm. Hg. is assumed inside the capillary, but outside the capillary there stand the tissue cells—an osmotic counterbalancing pull. Dilute the colloid of the blood with saline, and the water and salts might be taken up by the cells by osmosis from a low capillary pressure. Knowlton claims that if 5 per cent. of gelatine in Ringer solution is injected with an osmotic pressure of 23 mm. Hg.—a figure pretty nearly the osmotic pressure of blood colloid—there is no diuresis ; but on the modern theory the only means in such case of stopping an increased flow of urine would be to raise the osmotic pressure of the blood to 20 per cent. less than the arterial pressure, viz. 90 mm. Hg. !

It may be said that increased filtration through the glomerular capillaries does in fact take place, but the water and salts are what Cushny calls an optimal fluid, and so are absorbed in the tubules. Well, if such be the case, the modern view of kidney function expressly points out that the call for oxygen or increase of oxygen consumption follows on the absorption process, not the filtration process. Now it is pointed out by Knowlton that whether Ringer solution alone or Ringer solution *plus* 5 per cent. gelatine are injected the consumption of oxygen in each case is practically the same. In this experiment the modern view of glomerular function breaks down.

We may here note that Tamura and Miwa (9) find that physiological permeation of the kidney occurs without any increase of oxygen use.

To sum up, then, the diastolic pressure of small arteries in the mammalian kidney lies between 40–50 mm. Hg. The blood-pressure in the second capillary system lies close to the pressure observed by Burton Opitz in the renal vein (11 mm. Hg.). The capillary pressure in the glomeruli must lie between 40–50 mm. Hg. and 13–14 mm. Hg., and as a large drop of pressure exists elsewhere in the body between the small arteries and the capillary field, there is good reason to think that glomerular capillary pressure does not exceed 15–20 mm. Hg. The maximum secretory pressure depends on the compression exerted by the secretion in the tubules on the veins, capillaries and arteries of the kidney, and is determined by the diastolic pressure of the small arteries. When these arteries are compressed during diastole less blood gets through, venous outflow is diminished and the kidney cells cease to secrete.

The production of diuresis by saline injection and the failure of such by injection of saline *plus* 5 per cent. gelatine are against the filtration theory of production of urine, and suggest that diuresis depends on a physiological permeation. In the structure of the kidney there is no evidence of structure which would support the blood capillaries and allow them to act as a filtering membrane. Swelling of the tubules by compressing the venules will raise the pressure in the capillaries and venules up towards the arteriole pressure and cause a swift flow of blood through a more rigid tense system of vessels, the flow not being impeded until the diastolic arteriole pressure is overtopped.

The glomerular capsules and tubules are not empty spaces into which filtration can take place, but contain fluid. The pulse transmitted through the arteries expands the whole kidney confined within its capsule, and expresses this fluid towards the pelvis just as it impels venous blood out of the venules into the veins. Hence the relation of flow of urine to pulse pressure found by Erlanger and Hooker ([10, 11]) and by Gesell ([12]). The kidney enclosed by its capsule is comparable in this respect to the brain enclosed by the dura mater and cranial wall. We know that the blood pulses out of the cerebral venous sinuses and that the cerebro-spinal fluid, when connected with a gauge, shows similar pulses.

We now turn to experimental observations made by one of us (L.H.), who has observed the glomeruli of the frog's kidney under the microscope, watched the circulation and measured the glomerular capillary pressure.

Method.—A male frog is put under urethane, or has its skull crushed between the blades of artery forceps so as to destroy the brain, leaving the spinal bulb intact. The frog is stretched out on its side and a crucial incision made through the skin over the ilium on that side which is uppermost. The body-wall is cut through along the upper two-thirds of the ilium and this part of the bone removed. Any hæmorrhage is stopped by a cautery heated to a dull heat. The membrane at the outer edge of the kidney is then picked up in the forceps at a point about one-third above its lower end, and a ligature tied on there. The animal is then laid belly upwards on an apparatus similar to that designed by Graham Brown for the research which he carried out with Roy on capillary pressure. ([13]) The apparatus consists of a small cylinder of brass with a glass floor, and a dome-shaped piece of transparent peritoneal membrane tied on "atop." Into the cylinder there enters a tube which is connected by a ⊥-piece with (1) a manometer, (2) a tube for blowing air in and raising the pressure. The brass cylinder forms the centre of a flat piece of metal which is covered with a sheet of cork, to which cork the frog's head and knee-joints are pinned suitably. The outer border of the kidney, by means of the thread attached to it, is gently drawn on to the peritoneal membrane which covers the top of the brass cylinder. The kidney is then wetted with Ringer's solution, and a flat piece of thin glass, attached to a holder (forming a part of the apparatus) is lowered down so as to cover the kidney. The kidney can now be compressed between the peritoneal membrane and the glass cover when air is blown into the cylinder. The apparatus is brought on to the stage of the microscope, and the kidney examined with the low power.

The blood will be seen streaming from the renal portal vein in copious slow streams over the surface from the outer to the inner border. Between these vessels glomeruli may be seen, sometimes very clearly on the outer border. On compressing the kidney by 2–3 mm. Hg. the venous blood is expelled and the glomeruli come more clearly into view. The blood corpuscles can be seen swirling through the glomerular network; the capsules and the afferent and efferent vessels may be visible in some cases. The arterioles supplying the glomeruli may also be observed crossing the kidney in successful preparations. A pressure of 5–10 mm. Hg. suffices to slow distinctly the flow in the glomeruli, while it takes 20–35 mm. Hg. to stop the flow in the arterioles supplying them. The glomerular capillary pressure is about as high as that found in the papillæ of the frog's tongue, and only a little higher than that in the frog's web. It may be concluded, then, that the frictional resistance to the normal rate of flow in the glomerular capillaries is very small, just as it is found to be in other capillaries.

After injecting subcutaneously 1 c.c. of Ringer's solution, or normal saline, an hour before preparing the kidney for examination, the capsules are found distended with clear fluid equal to or even double the volume of the glomerulus. As it still takes a pressure of some 6 mm. Hg. to retard the glomerular circulation, it follows that the fluid distending the capsule is at a less pressure than this, and that it takes very little pressure to distend the capsule, which must be an easily distended structure. It follows, too, from these observations that the glomeruli are in truth the seat of physiological permeation or secretion of watery fluid, as has been generally surmised on other grounds, and that the fluid may come through much quicker than it passes away down the tubules. The latter do not appear distended with fluid. It is worth noting that the distended capsules are surrounded with capillary networks in which flows the blood from the renal portal veins. It seems possible that some interchange may go on between the intra-capsular fluid and the venous blood.

We may here note that Hamburger (14) claims that the glomerular permeability is physiological and selective. Perfusing the frog's renal arteries with Ringer solution, he finds a retention of glucose (added to the Ringer in the normal concentration pertaining to the blood) depending on the composition of the Ringer solution. If potassium ions are left out, for example, the glomerular epithelium becomes permeable to glucose. Another radio-active element can replace potassium just as Zwaardemaker claims to be the case with the frog's heart. While the glomerular epithelium retains glucose, it is permeated by levulose and other sugars.

CONCLUSIONS.

The paper deals with the modern theory of the secretion of urine, and evidence is brought forward to show that pressure in the glomerular capillaries is a low one, and that physiological permeation—not filtration—controls the passage of fluid through the glomeruli. Observations on the circulation in the frog's glomeruli are detailed and support these conclusions, which are also borne out by experiments of Hamburger, which demonstrate the selective action of the epithelium covering the glomeruli.

REFERENCES.

1. Cushny, A. R.—'The Secretion of Urine,' London, 1917. (This work contains references to authors cited other than those given below.)
2. Opitz, R. B.—Cited by R. Tigerstedt, "Die Strömung des Blutes in den Kapillaren und Venen," *Ergebn. d. Physiol.*, 1920, **18**, 10.
3. Hill, L.—"The Capillary Blood-Pressure," *Proc. Physiol. Soc.*, July 10th, 1920; December 18th, 1920; February 21st, 1921; *J. Physiol.*, 1920, **54**, xxiv.
4. Hill, L., and McQueen, J.—"The Measurement of Capillary Blood-Pressure in Man," *Brit. J. Exper. Pathol.*, 1921, **2**, 1.
5. Basler, A.—"Untersuchungen über den Druck in den kleinsten Blutgefässen der menschlichen Haut," *Arch. f. d. ges. Physiol.*, 1912, **147**, 393.

6. References given by Tigerstedt, *vide* 2 *supra*.
7. Flack, M., Hill, L., and McQueen, J.—"The Measurement of the Arterial Pressure in Man," *Proc. R. Soc.*, 1914–15, **88** b, 525.
8. Henderson, V. E.—"The Factors of the Ureter Pressure," *J. Physiol.*, 1905, **33**, 175.
9. Tamura, K., and Miwa, M.—"Über den Sauerstoffverbrauch der Niere," *Mittl. Fak. Univ. Tokyo*, 1912, **12**, 317, 349.
10. Erlanger, J., and Hooker, D. R.—*Johns Hopkins Hosp. Rep.*, 1904, **12**, 346.
11. Hooker, D. R.—"The Influence of Pulse-Pressure upon Renal Function," *Amer. J. Physiol.*, 1910, **27**, 24.
12. Gesell, R. A.—"The Relation of Blood-Pressure to the Renal Secretion," *ibid.*, 1913, **32**, 70.
13. Roy, C. S., and Brown, J. G.—"The Blood-Pressure and its Variations in the Arterioles, Capillaries and Smaller Veins," *J. Physiol.*, 1879–80, **2**, 323.
14. Hamburger, H. J.—"La perméabilité partielle de la membrane glomérulaire, etc.," rev. *Arch. Neerland. de Physiol.*, 1920, **4**, 582, 586, 588.

THE THEORY OF AGGLUTININ ESTIMATION.

J. O. WAKELIN BARRATT, M.D., D.Sc.

From the Lister Institute, London.

Received for publication July 28th, 1921.

In the present communication the mathematical expression of (1) the action of agglutinin upon antigen, and (2) the decay of antigen, is considered in relation to the estimation of agglutinin.

THE AGGLUTININ-ANTIGEN REACTION.

I. The reaction between agglutinin and its antigen (bacteria, red cells, blood-platelets, etc.) was observed (Arrhenius (1905), on the basis of experiments at 37° C. by Madsen) to be in fair agreement with the equation—

$$yt = m \quad . \quad . \quad . \quad \text{(I)}$$

y representing the concentration of the agglutinin-holding serum employed, t the time of appearance of agglutination, and m being a constant. The curve given by this equation is a rectangular hyperbola (Fig. 1). The reaction recalls that of hydrochloric acid upon a marble surface: so long as the area of the surface remains unaltered and the amount of acid present is not appreciably changed equation (I) is applicable, the time required for a fixed amount of marble to dissolve being inversely proportional to the concentration of acid.

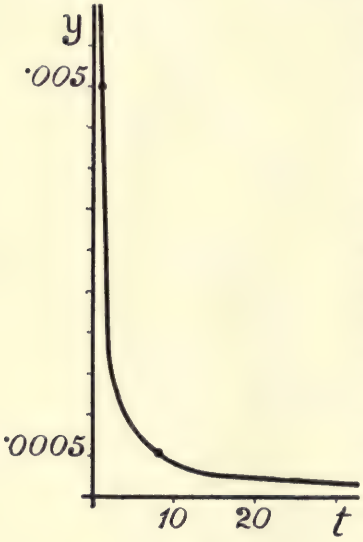


Fig. 1.

McKendrick (1911), in the course of a study of amboceptor action, reviewed the experiments quoted by Arrhenius, and advanced the view that the reaction between agglutinin and its antigen in reality proceeds at a bimolecular reaction rate, represented by the equation—

$$yt = \frac{c^2 z}{cz - y} \quad . \quad . \quad . \quad . \quad . \quad \text{(II)}$$

where y and t have the same significance as before, while c and z are constants. If two experiments are made with concentrations of serum

y_1 and y_2 and times of appearance of agglutination t_1 and t_2 respectively, then—

$$c = \frac{y_1 y_2 t_1 t_2 (y_1 - y_2)}{y_1^2 t_1 - y_2^2 t_2} \quad . \quad . \quad . \quad . \quad \text{(III)}$$

and—

$$z = \frac{y_1^2 t_1}{c\,(y_1 t_1 - c)} = \frac{y_2^2 t_2}{c\,(y_2 t_2 - c)}. \quad . \quad \text{(IV) and (V)}$$

The curve corresponding to equation (II) is of the type shown in Fig. 2. The action, it will be observed, ceases when a critical concentration is reached (indicated in the figure by the upper asymptote), in this respect recalling that sometimes exhibited by high concentrations of powerful agglutinating sera, which, as is well known, may fail to give rise to agglutination until a dilution of, it may be, 1 in 50 or more is reached.

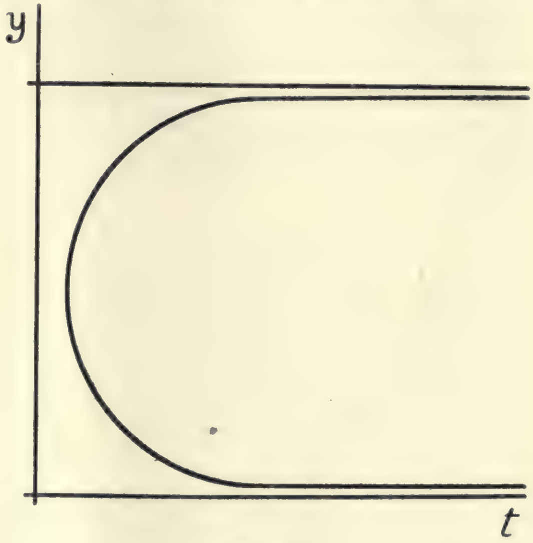


FIG. 2.

The two series of experiments quoted by Arrhenius are given in Table I.

The amounts (in c.cm.) of agglutinating serum employed are indicated by y and the observed agglutination periods (in minutes) by t; in the third column t_1 represents the agglutination periods calculated, according to equation (I), for $yt = 0·995$ in the first series and $yt = 0·896$ in the second; in the fourth column t_2 gives the same periods calculated, according to equation (II), for $c = 0·92$ and $z = 0·208$ in the first series and for $c = 0·90$ and $z = 0·046$ in the second.

TABLE I.

y	t	t_1	t_2	y	t	t_1	t_2
0·080	[20]	12	20	0·008	[140]	112	139
0·035	30	28	32	0·006	150	149	175
0·025	45	40	42	0·0055	165	163	188
0·017	60	58	60	0·005	180	179	204
0·012	90	83	82	0·004	210	224	249
0·008	120	124	120	0·0035	240	256	281
0·005	180	199	189	0·003	300	299	323
0·004	240	249	235	0·0022	420	407	432
0·003	300	332	312	0·002	480	448	472
0·0027	360	369	346				

It is seen that the calculated periods approximate closely to the observed, no matter which of the two equations given above is employed. The experiments do not, however, afford a fair test of the applicability of equation (II), the values of y employed being small compared with cz ($= 0{\cdot}1915$ in the first series and $= 0{\cdot}0414$ in the second), so that, for these values, the expression to the right of (II) becomes approximately constant and the equation thus resembles (I). With higher concentrations of agglutinin y ceases to be negligible compared with cz, and it becomes possible to determine if the reversal of the curve, shown in Fig. 1, occurs. It is found that experiments exhibiting a wide range of values of y not only fail to give evidence of reversal, but in addition show marked clumping with concentrations of agglutinin exceeding the limiting value at which, according to equation (II), the period of agglutination should become indefinitely prolonged. This is illustrated in the series of observations given below in which *B. dysenteriæ* (Flexner) was agglutinated by rabbit's anti-serum:

TABLE II.

y	t	t_1	t_2
0·5	< 0·20	—	—
0·05	< 0·20	—	—
0·0375	0·25	0·045	0·29
0·0025	0·67	0·68	0·72
0·00075	2·4	2·3	2·3
0·00025	6·5	6·8	6·8

Within the range of concentration of agglutinin covered by the last three experiments both equations are applicable, the calculated periods t_1 and t_2 being found to agree closely with those actually observed (the constants selected were—for equation (I) $yt = 0{\cdot}0017$; for equation (II) $c = 0{\cdot}0017$, and $z = 26$). With a concentration of antiserum represented by 0·0375 accurate determination of the period of agglutination became difficult; with still higher concentrations very marked agglutination occurred with a rapidity too great for measurement. But according to equation (II) agglutination ceases when an agglutinin content corresponding to 0·0442 c.cm. of anti-serum contained in 1 c.cm. of fluid is reached: it is obvious, therefore, that this equation fails to represent the relation between concentration of agglutinin and period of agglutination, although in the last three experiments, where y is small compared with cz, the agreement between observed and calculated results is close.

The equation of Arrhenius does not imply any limitation in respect of the occurrence of agglutination when the content of agglutinin is high. Its limits of applicability are chiefly determined, on the one hand, by the diminution in degree and consequent ill-defined character of agglutination with increasing dilution of anti-serum, and, on the other, by the extreme rapidity with which clumping takes place when sufficiently high concentrations of agglutinin are used. Within these limits, which must be ascertained experimentally, equation (I) is valid.

ESTIMATION OF AGGLUTININ.

II. The existence of three variables is implied in equation (I), namely agglutinin (y), time (t) and "effect" (agglutination). The latter is not met with in the equation, but the constant (m) represents a function, not yet determined, of the "effect," and only appears as a constant owing to the present impossibility of estimating quantitatively degrees of "effect." If two of these variables, *e.g.* time and "effect," are measured in fixed units, then the third variable, agglutinin, can be expressed in terms of the first two and is thus obtained in absolute measure. In selecting degree of "effect" only one choice is possible, namely the smallest amount of agglutination which can be recognised. The unit of "effect" is represented by this degree of "effect" occurring in presence of (1) a fixed amount of antigen exhibiting (2) a known degree of agglutinability (both are dealt with further below). If the procedure generally adopted in selecting physical units is followed and the constant in equation (I) is made equal to unity, then the unit of agglutinin is given by the equation—

$$yt = 1 \quad . \quad . \quad . \quad . \quad . \quad . \quad \text{(VI)}$$

A serum (undiluted) producing unit "effect" in unit time (1 min.) would thus contain one unit of agglutinin in unit volume (1 c.cm.). If for any other serum $yt = x$, then the normality of this serum is $1/x$. It may be pointed out that the unit of "effect" will not be the same when determined with the naked eye as it is when observed under the microscope, the degree of agglutination required for liminal observation being greater in the former case than in the latter. The ratio of the two units may be determined by making comparative estimations of agglutinin with the same serum. A certain range of variability of result among different observers is to be anticipated, however, since the least amount of agglutination which can be perceived depends upon (1) the acuity of the observer's vision and (2) his power of discrimination, both of which vary in different individuals. Moreover no sharp transition point is met with between an unchanged suspension and one in which agglutination is just recognisable: this applies equally whether agglutination is observed (1) macroscopically at the end of a given period of time, in a graduated series of dilutions of serum, or (2) microscopically, a single dilution being used and the time at which clumping first becomes recognisable noted.

DECAY OF ANTIGEN.

III. The estimation of agglutinin in absolute measure implies, as indicated above, that the antigen employed has an ascertained agglutinability. It is found, however, that both the agglutinin content of a serum and the agglutinability of a bacterial suspension undergo change on keeping. If given amounts of a suspension of bacteria are added at intervals of a few days to samples of a corresponding agglutinating serum (the same quantity of the latter being used in each experiment), it is usually found that the agglutination period increases with lapse of time. If the age (in days) of the serum and suspension, reckoned from the commencement of the experiments, is plotted as ordinate against the period (in minutes) of agglutination as abscissa, a curve similar to that shown in Fig. 3 is obtained. The lengthening of the time of appearance of agglutination with increasing age of serum and suspension is at first relatively considerable and later becomes relatively slight. As soon as the rate of change has become sufficiently small it is obvious that if now a new serum is prepared, the rate at which this changes on keeping may be determined with a fair degree of accuracy by ascertaining its agglutination period at varying intervals, employing for this purpose the old suspension of bacilli; and conversely, the rate of change of a new suspension may similarly be studied with the aid of the old serum, provided of course that in each case the rate of change to be determined is much greater than that still observable in the course of additional experiments, made with the old serum and the old suspension, designed to explore the further course of the curve given in Fig. 3. The change undergone by an agglutinating serum on keeping will not be considered here; that occurring in a bacillary suspension will alone be dealt with. Some observations upon a freshly prepared suspension of a strain of *B. dysenteriæ* (Flexner) in 0·85 per cent. NaCl solution, containing $1{\cdot}4 \times 10^9$ bacilli per cubic centimetre, were carried out, at 16°C., with the aid of an agglutinating serum, thirteen months old, made from this strain; these are given in Table III and are shown graphically in Fig. 3. Owing to the age of the serum it would appear likely, in view of the type of curve obtained, that the increase of the period of agglutination occurring on keeping is attributable principally to deterioration of the suspension of bacilli. It was found that the observed periods of agglutination at the end of 0, 10 and 26 days were the same, within the limits of experimental error, when the observations were made simultaneously with bacillary suspensions 0, 10 and 26 days old respectively, as when consecutive observations were made upon the same suspension at the end of the same periods of time. In the observations recorded in Table III, therefore, no recognisable fraction of the agglutination period is attributable to deterioration of the serum during the period of experiment; the increase of this period with lapse of time thus appears to be due solely to deterioration of the suspension.

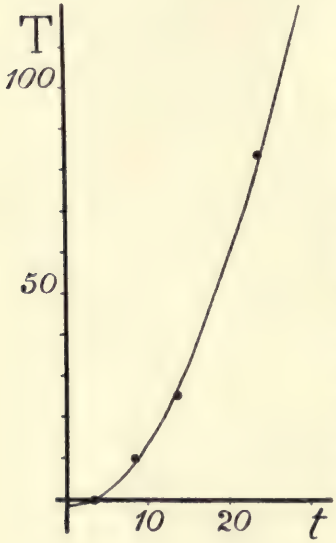


Fig. 3.

TABLE III.

Age of suspension T.	Dilution of serum.	Bacilli per 1 c.cm.	Agglutination period t.
0 days	1 in 2000	$1{\cdot}4 \times 10^9$	3·4 min.
10 ,,	,,	,,	7·5 ,,
26 ,,	,,	,,	14·9 ,,
83 ,,	,,	,,	23·0 ,,

The experiments of Table III, shown in Fig. 3, seem to lie upon a parabolic curve. The equation for a curve of this type is—

$$T - a = bt^2 \quad . \quad . \quad . \quad . \quad . \quad \text{(VII)}$$

where T represents age of suspension, time of appearance of agglutination, while a and b are constants. If this relation between T and t is assumed to be valid and the values of a and b are taken at $-1\cdot5$ and $0\cdot15$ respectively, then the values of t at varying intervals after preparation of the suspension may be calculated. A comparison of observed and calculated values is shown below:

TABLE IV.

T.	$t_{calc.}$	$t_{obs.}$
0 days	3·2 min.	3·4 min.
10 ,,	8·7 ,,	7·5 ,,
26 ,,	13·5 ,,	14·9 ,,
83 ,,	23·7 ,,	23·0 ,,

Since a fair correspondence exists between observed and calculated values equation (VII) may be regarded as valid for the range investigated. The curve exhibits the varying sensitiveness of the suspension at different periods after preparation. The constant a indicates the point upon a theoretical curve at which the examination may be imagined to commence: in other words it represents the equivalent age of the suspension at the actual beginning of experiment. So long as the concentration of agglutinin is unchanged the constant b represents the agglutinability of the suspension, so far as this is exclusively an individual character of the culture used; if, on the other hand, the agglutinability of the suspension remains unaltered, then b is (by equation (I)) proportional to the square of the concentration of serum (agglutinin) employed.

STABLE ANTIGEN.

IV. So long as the agglutination period of a suspension, tested at intervals against an old serum, is observed to be undergoing change, doubt arises as to its stability, and any estimations of agglutinin made with it when the rate of change is small cannot be regarded as possessing more than a comparative value. A fixed agglutination period may be obtained (cp. Dreyer (1906); Gardner (1918)) if the bacteria have been subjected to the action of fixing agents (heat, formaldehyde, thymol, chloroform, phenol). These usually produce at first a diminished sensitiveness, indicated by a lengthening of the agglutination period, sometimes considerable (50 per cent. or more), occasionally absent, after which the period becomes stationary. In some cases the stationary period is again followed by an increase in sensitiveness, indicated by a shortening of the agglutination period. The circumstance that a suspension of bacteria may on keeping exhibit an increase of sensitiveness renders difficult the interpretation of a stationary agglutination period: this may be due either to stability of both suspension and serum, or it may be that a diminution of the agglutinin content of the serum is occurring, but is compensated by an increasing sensitiveness of the suspension. No evidence of a late increase of agglutinability has, however, been observed with formalised suspensions, which are regarded as stable when exhibiting a stationary agglutination period.

AMOUNT OF ANTIGEN EMPLOYED.

V. It has already been stated that in order to define unit of "effect" it is necessary that the antigen employed should be present in fixed amount. In all the experiments given in this article the suspension used contained $1{\cdot}4 \times 10^9$ bacteria per cubic centimetre, observations being made with dark background illumination and a magnification of 350 diameters. Variation of bacterial content, unless extreme, was not found to affect markedly the agglutination period, as is exhibited by the following experiments carried out at 16°C.

TABLE V.

Dilution of serum.	Bacilli per 1 c.cm.	Agglutination period.
1 in 2000	$1{\cdot}4 \times 10^9$	15 min.
,,	,,	14·5 ,,
,,	,,	15·5 ,,
,,	,,	15 ,,
,,	,,	14·5 ,,
,,	$\cdot 14 \times 10^9$	12 ,,
,,	,,	13 ,,
,,	,,	9 ,,
,,	,,	10 ,,
,,	,,	10·5 ,,
,,	,,	10·5 ,,

INFLUENCE OF TEMPERATURE.

VI. Agglutination proceeds more rapidly with rise of temperature, a maximum being reached at 55°C. (Dreyer). In experiments carried out as in Table III, the agglutination periods at 0°C., 16°C. and 38°C. were 60 min., 15 min. and 5 min. respectively. Variation of 1°C. above or below 16°C. affected the agglutination period by ± 6·2 per cent.

SIGNIFICANCE OF AGGLUTININ DETERMINATION.

VII. In agglutination determinations only that fraction of the total agglutinin content of the serum tested is estimated which corresponds to the particular strain of bacteria used for the suspension. For the estimation of the fraction resulting from immunisation, therefore, a suspension of the particular strain actually used for immunisation can alone be employed; other strains, which may give widely varying results, or even fail to agglutinate, do not constitute a natural standard of reference. Different immune sera can be compared in respect of their agglutinin content only when immunisation has in each case been produced by the same strain. This condition may be fulfilled in experimental work; its fulfilment when dealing with strains isolated from cases of naturally acquired infection cannot be asserted with any degree of certainty, owing to the circumstance that although grouping of strains may be possible, it is nevertheless at present impossible to establish identity of strain.

ABSOLUTE MEASURE.

VIII. Only one observer has, up to the present, attempted to estimate agglutinin in absolute measure, namely Dreyer (1906), who has selected as unit of effect "marked agglutination without sedimentation," observed with the naked eye, and as unit of time 2 hours in the case of *B. typhosus* (*B. dysenteriæ* 4·5 hours), the temperature of experiment being 55° C. A formolised bacterial suspension, exhibiting a stationary agglutination period when tested with a fixed amount of an old agglutinating serum and containing from 3×10^8 to 1×10^9 bacilli per c.cm., is selected as antigen, all other suspensions being standardised in terms of this and a correction co-efficient determined for each. In this method only a single point on the y,t curve, Fig. 1, is investigated, the agglutinin content of the concentration of serum corresponding to this point being, by definition, normal. The reciprocal of this concentration represents the normality of the undiluted serum.

ESTIMATION BASED ON VALUE OF b.

IX. It may happen that the agglutinability of a strain of bacteria will be found on keeping always to diminish in the same manner, according to equation (VII), a suspension containing a fixed number of bacteria per c.cm. when tested against an old serum always giving the same value of b and exhibiting the same curve, of the type shown in Fig. 3. In such a case—and it is to be anticipated that strains will be met with exhibiting this type of specific decay of antigen in the presence, or it may even be in the absence, of fixed conditions of culture and mode of preparation of suspension—it becomes possible to determine in absolute measure the agglutinin content of any old serum, prepared from the strain in question, by determining the value of b for a given dilution of the serum. If that dilution of serum for which $b = 1$ is defined as normal, then the normality of any dilution for which $b = x$ is $\sqrt{x}$, whence the normality of the undiluted serum may be determined. By means of comparative tests with the latter the agglutinin content of a fresh (labile) serum may be ascertained. [The determination of b may also be of value in the identification of strains.] If the strain employed in Table IV were of the type under consideration the normality of the 1 in 2000 dilution of serum would be $\sqrt{0{\cdot}15}\ N = 0{\cdot}387\ N$ and that of the undiluted serum 774 N.

NATURE OF ANTIGEN DECAY.

X. If it is assumed—as is not inherently improbable—that, in a suspension of bacteria, the available antigen, which may be represented by C, is inversely proportional to t, then equation (VII) may be written—

$$T = \frac{B}{C^2} + a \quad . \quad . \quad . \quad . \quad . \quad \text{(VIII)}$$

T and a having the same signification as before, while B is a new constant replacing b. Differentiating we obtain—

$$\frac{dC}{dT} = -kC^3 \quad . \quad . \quad . \quad . \quad . \quad \text{(IX)}$$

where k is a constant $\left(= \frac{1}{2B}\right)$. In this form the decay of antigen appears as a condensation or polymerisation process.

SUMMARY.

(1) For a given "effect" the action between agglutinin and antigen proceeds according to the equation, applicable to a surface action, given by Arrhenius—

$$yt = m,$$

y being concentration of agglutinin, t agglutination period and m a constant.

(2) Using a strain of *B. dysenteriæ* (Flexner), decay of antigen was found to be represented by the equation—

$$T - a = bt^2,$$

T being age of suspension of bacilli, t, as before, agglutination period, a and b constants.

(3) The relation between y and t given in the first equation is applicable to the estimation of agglutinin in absolute measure if the antigen employed is stable; with antigen subject to decay absolute determinations of agglutinin are still possible for a suitable type of constant b, given by the second equation.

REFERENCES.

Arrhenius, S.—(1905) 'Immuno-Chemistry,' p. 68.

Dreyer, G.—(1906) Cp. *Med. Res. Council, Spec. Rep. Ser.*, No. 51, London, 1920 (H.M. Stationery Office).

Gardner, A. D.—(1918) "The Standardisation of Killed Agglutinable Cultures of *B. dysenteriæ*," *J. Hyg.*, **17**, 471.

McKendrick, A. G.—(1911) "The Chemical Dynamics of Serum Reactions," *Proc. R. Soc.*, B, **83**, 493.

THE PREVENTIVE EFFECT OF INFLUENZA VACCINE.

S. YABE.

Kitasato Institute for Infectious Diseases, Tokyo.

Received for publication July 22nd, 1921.

On the outbreak of the last pandemic of influenza (October, 1918–March, 1919) we first studied the cause of the disease by bacteriological, pathological-anatomical, hæmatological and serological methods, and came to the conclusion that the pandemic was due to Pfeiffer's influenza bacillus, and that the complications were very often caused by various cocci, especially by pneumococci. An account of this work has been published by my colleague, Dr. Okawara, and some co-workers (1918). I continued the study of the immunity to mice, guinea-pigs and rabbits.

Although the toxicity of influenza bacilli is rather strong as compared with the antigenic property, we can render animals immune to a certain degree. Influenza bacilli differ particularly from ordinary pathogenic bacilli in that animals, while rendered moderately immune, are intensely weakened, and even the maximum stage of immunity is not so strong as in the case of other bacilli. This accounts for the fact that a relapse often occurs in human influenza, and that the immune reactions in convalescent serum are slight. From the result of my experiments I could not expect to find that vaccination had as great a preventive effect against influenza as it had against typhoid fever or cholera asiatica. Statistics, however, show that the majority of people who have recovered from influenza are not again attacked by it for a few months, although the amount of immune body in their serum is slight. In view of these facts a slight resistance of a special type might be sufficient to prevent the infection of influenza, and this appears more likely when one considers that there are so many carriers of influenza bacilli during the epidemic, and that real attacks of this disease are occasionally caused by some bodily weakness. I have inoculated three kinds of vaccine, but this report deals only with those cases inoculated with heated vaccine. This was made as follows: 18–20 hours' growth on heated blood agar was emulsified in saline in the proportion of 5·5 mgrm. to 1 c.c. and heated to 60° C. for half-an-hour. When cold this suspension was diluted with one-ninth of its volume of 5 per cent. carbolic saline. When the vaccine was to be bottled it was diluted 9·9 times with carbolic saline, having thus 0·5 mgrm. per c.c. Two inoculations were usually made at an interval of four days subcutaneously in the interscapular region. The first dose for an adult was 0·5 c.c. and the second 1 c.c. These quantities and intervals were determined by experimentation upon myself and others, regard being directed to the bodily reaction and the decrease in leucocytes which facilitates infection.

CONDITIONS NECESSARY FOR JUDGING THE PREVENTIVE EFFECT OF VACCINATION.

To determine the effect of vaccination the conditions under which observation is made should be carefully examined. For statistical purposes the three essential conditions are time, locality and person. In order that full immunity may exist at the outbreak of the epidemic the vaccination should be completed about a fortnight before the outbreak. Owing to the negative phase, as shown by the leucocyte count, a vaccinated person would be more susceptible than a normal if the vaccination was completed just at the beginning of the epidemic, or a few days previously. Vaccination begun during or at the end of an epidemic does not test its preventive effect, since those inoculated would be the residuum of strong people naturally immune.

Regarding locality, care should be taken to select an area which had not been affected at the time of the vaccination.

The personal condition should be strictly observed. The uninoculated control person and the inoculated should live in the same place—for instance in a dormitory. They should be of the same age and sex, with identical occupations, so that their liability to infection may be equally great. For the statistics of a second epidemic, those attacked in the first epidemic should be omitted when considering the preventive effect of the vaccines which were inoculated before the second epidemic.

Thus there are not many good opportunities for determining the preventive effect of vaccination, since most are lacking in one or more of the required conditions. Vaccination with my influenza vaccine was adopted so widely that at the end of the second epidemic (March, 1920) the number of inoculated persons amounted to over half a million. Many variable statistics were reported to me by doctors in the city and in the country. Unfortunately, however, these results were always lacking in some of the conditions necessary for determining the effect of the vaccine. It would be better here to report our own cases in which the three conditions above mentioned were perfectly observed, and to add another series on a different plan from ours, which was examined by my friend, a doctor in the preventive medicine department of a hospital for the families in the Hidachi mining district.

THE RESULT OF VACCINATIONS IN NAGOYA CITY.

In our case, the inoculation was made in two cotton factories in the environs of Nagoya City, one belonging to the Toyoda Company, the other to the Kikuye Company. These two factories are controlled by the same staff. Each have their own workshops, dormitories, laundries and baths, also a power-house, a store, a shop for daily necessaries, a hospital and a school. All these buildings are encircled by a high wall. The clerks, business men, female spinners and weavers, the last two of which constitute over 90 per cent. of all those inoculated, live and work in this enclosure. The female workers are not allowed to go out more than twice a month. As a matter of fact they used to go out once a month. Neither children nor old men live there. In the hospital there are two private doctors with nurses under a matron, entirely at the service of the inmates. We divided these persons into two parties; one was inoculated, the other was left uninoculated as a control.

The personal conditions regarding sex, age, work, mode of life and the prevention of external communication were thus well fulfilled—that is to say, exposure to the danger of infection was about the same in the inoculated party and in the uninoculated control party. As all were living in the same enclosure, having little opportunity for external communication, the local conditions may be approved. As to the condition of time, the intervals between the inoculation and the outbreak of the second epidemic in these two factories and in the surrounding district were as follows:

Days between Inoculation and Epidemic in the Toyoda Factory and in the Surrounding District.

Inoculation.	Date of inoculation.	Epidemic in Toyoda factory.	Epidemic in the surrounding district.
I	December 25th–26th, 1919	Began on January 13th, 1920	Began about the end of December, 1919.
II	December 28th–29th, 1919	Ended at the end of January, 1920	Ended about the beginning of March, 1920.

Days between Inoculation and Epidemic in the Kikuye Factory and in the Surrounding District.

Inoculation.	Date of inoculation.	Epidemic in Kikuye factory.	Epidemic in the surrounding district.
I	December 24th–25th, 1919	Began on January 8th, 1920	Began about the end of December, 1919.
II	December 29th–30th, 1919	Ended on January 28th, 1920	Ended about the beginning of March, 1920.

TABLE I.—*The Number of Patients in the Inoculated and Uninoculated Parties in the Factory belonging to the Toyoda Company from December*, 1919, *to March*, 1920.

	Twice inoculated.	Once inoculated.	Not inoculated.	Total.
Number	2075	152	237	2464
Typical influenza	40 (1·93)	8 (5·26)	8 (3·38)	56 (2·27)
Influenza combined with catarrhal pneumonia	3 (0·14) (2 died)	1 (0·65)	2 (0·84) (1 died)	6 (0·24) (3 died)
Per cent. of catarrhal pneumonia to all influenza	(6·97)	(11·11)	(20·00)	(9·68)
Acute bronchitis	57 (2·75)	10 (6·25)	13 (5·49)	80 (3·24)
Pharyngitis	10 (0·48)	2 (1·32)	1 (0·37)	13 (0·52)
Laryngitis	1 (0·05)	0	0	1 (0·04)
Tonsilitis	1 (0·05)	2 (1·32)	1 (0·34)	4 (0·16)
Lobular pneumonia	0	1 (0·65)	0	1 (0·04)
Total number of cases with illnesses of the respiratory tract	111 (5·40)	24 (15·79)	25 (10·55)	161 (6·51)

(The figures in brackets show the percentage of the number of persons in each category of inoculation.)

We observed the cases throughout the second epidemic, and recorded those of influenza and all other illnesses apparently related to this disease which occurred between December 24th–25th, 1919 (the first day of inoculation) and March 10th, 1920. In Table I the persons who were attacked by influenza during the first epidemic are not taken into account.

Chart showing the Number of Illnesses of the Respiratory Tract as above per month, together with the Total Number in the Enclosure from December, 1917, *to February,* 1920.

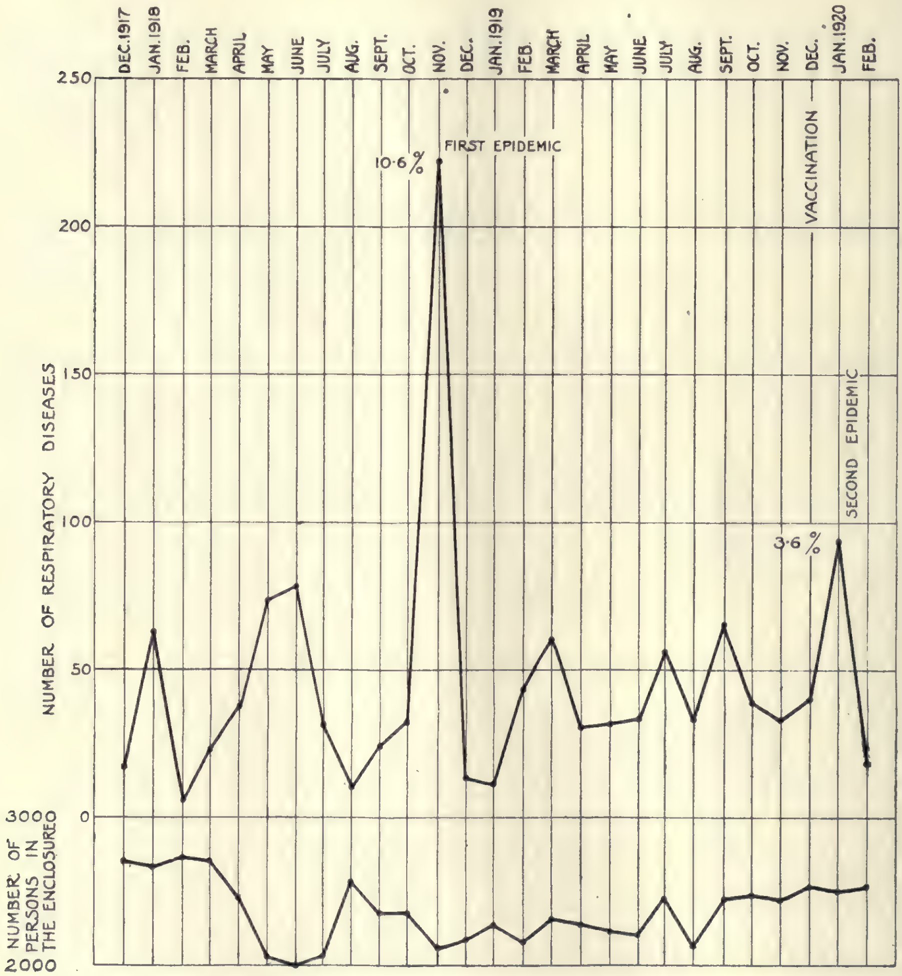


Table II.—*The Number of Typical Cases of Influenza in the Inoculated and Uninoculated Parties in the Kikuye Factory during the Second Epidemic.*

	Number.	Typical cases, including catarrhal pneumonia.		Influenzal catarrhal pneumonia.			Deaths.			Average length of fever.
		Number.	Per cent. of total.	Number.	Per cent. of patients.	Per cent. of total.	Number.	Per cent. of patients.	Per cent. of total.	
Twice inoculated	1168	78	(6·68)	17	(21·82)	(1·46)	2	(2·56)	(0·17)	7·27 days.
Once inoculated	447	26	(5·82)	10	(38·46)	(2·24)	1	(3·85)	(0·22)	8·19 ,,
Not inoculated	235	42	(17·87)	16	(38·10)	(6·81)	2	(4·76)	(0·85)	9·05 ,,
Total	1850	146	(7·49)	43	(29·45)	(2·32)	5	(3·42)	(0·27)	8·17 days.

Table III.—*Cases slightly affected with Fever, having Acute Pharyngitis, Laryngitis, Tracheo-Bronchitis, which seemed to be Non-typical Influenza. The highest degree of fever in these cases was* 38°C.

	Number.	Non-typical cases.		Average length of fever.
		Number.	Per cent.	
Twice inoculated	1168	11	(0·94)	3·27 days.
Once inoculated	447	10	(2·24)	3·20 ,,
Not inoculated	235	10	(4·26)	3·60 ,,
Total	1850	31	(1·68)	3·35 days.

When the number of patients during the first epidemic is compared with the number of those during the second epidemic the difference is very remarkable. In the neighbourhood outside this factory it is alleged that the second epidemic attacked nearly half the persons who had escaped from the first epidemic. Inside this factory, however, all the above-named illnesses, most of which were influenza, amounted to 10·6 per cent. in the first epidemic (November, 1918), while in the second epidemic (January, 1920), after the preventive inoculation, they reached barely 3·6 per cent. In this percentage 55 remaining persons, who were attacked during the first epidemic, are excluded (*vide* Chart, p. 226).

In the Kikuye Factory there was a fairly large number of cases of influenza having typical symptoms and course. A small number of patients, however, were only slightly ill from some respiratory inflammation. We thus divided them into two groups, one typical, the other atypical (Tables II and III).

In the typical, as in the non-typical influenza, our statistics show that the rate of morbidity of those inoculated is decreased by about a half or a third, though there are some exceptions noted in detail. The most disagreeable complications from catarrhal pneumonia are very remarkably lessened, showing 0·14 per cent. as against 0·84 per cent. in the Toyoda factory and 1·46 per cent. to 6·81 per cent. in the Kikuye factory. I think this is a most interesting point—that the pure influenza vaccine can prevent catarrhal pneumonia and other inflammations of the respiratory tubes which accompanied the epidemic of influenza. But since the so-called catarrhal pneumonia develops only in the course of essential influenza, and since also other inflammations of the respiratory tubes may be due to influenza bacilli, the less frequent appearance of all these sequelæ in persons inoculated with the pure influenza bacilli would be expected. The average length of fever in typical and non-typical cases is always a little shorter among inoculated patients than among those uninoculated.

To compare the difference in the mortality, there are not, I consider, sufficient cases.

THE RESULT OF VACCINATIONS IN THE HIDACHI MINING DISTRICT.

Next I will quote statistics kindly offered by my friend. In this case the vaccination was begun in the Hidachi mining district just at the beginning of the second epidemic. The relation of the time of the inoculation to the course of the epidemic is as follows: The first case of typical influenza was observed on January 17th, 1920, and the last case on April 15th, 1920. The inoculation was begun three days after the appearance of the first case of influenza and continued on towards the end of the epidemic. Sixty-five per cent. of all the inoculations were made in the first 30 days after the onset of the epidemic, 28 per cent. were made in the next 20 days, the remaining 7 per cent. were inoculated in the last 10 days. Thus the inoculation continued for 60 days, having been begun on the third day after the onset of the epidemic. The epidemic, however, reached its acme between the fortieth and fiftieth day after its outbreak, and lasted to the ninetieth day.

Date.	Cases of influenza in every ten days.	No. of deaths from influenza.	Number of inoculations.		
Jan. 17	1	0			
,, 27	35	0	First inoc.	8484	
Feb. 6	123	1	Second inoc.	7098	
,, 16	185	11			
				15582	(65 per cent. of all inoculations).
,, 26	268	12	First inoc.	4689	
Mar. 8	214	15	Second inoc.	2042	
				6731	(28 per cent. of all inoculations).
,, 18	130	12	First inoc. Second inoc.	1078 549	
				1627	(7 per cent. of all inoculations).
,, 28	58	3			
April 7	30	8			
,, 17	9	1			

The number of inoculated and non-inoculated patients in this mining district was as follows, including the persons who had been attacked in the first epidemic or in the second epidemic before their inoculation :

TABLE IV.—*Number and Percentage of Influenza Cases Complicated by Catarrhal Pneumonia among all the Inoculated and Uninoculated Persons in the Hidachi Mining District.*

	Non-inoculated.	Once inoculated.	Twice inoculated.	Total.
Number of persons	12,501	4,562	9,689	26,752
Number of patients	810	132	116	1,058
Percentage affected	6·4	2·9	1·2	3·9
Number with catarrhal pneumonia complications	130	11	8	149
Percentage of catarrhal pneumonia to patients	(16·1)	(8·3)	(7·0)	(14·2)
Percentage of catarrhal pneumonia to persons	(1·04)	(0·24)	(0·08)	(0·56)
Deaths after complication with catarrhal pneumonia	52	3	1	56
Percentage of deaths to the number of complicated cases	(40·0)	(27·3)	(12·5)	(37·6)
Percentage of deaths to patients	(6·5)	(2·3)	(0·9)	(5·3)

In these figures there are many objections: (1) The persons who had been attacked during the first epidemic were not excluded ; (2) The persons who were attacked just before, or a few days after inoculation were not excluded.

To determine the preventive effect of the vaccine the statistics should be begun at the completion of immunity, excluding persons previously attacked and also those who were attacked during the negative phase after the vaccination.

In order to compare exactly the number of the inoculated patients with those uninoculated, we limited the statistics to certain families attacked by the second epidemic and excluded persons who had been attacked during the first epidemic. The following results were thus obtained. The families here referred to consist of those whose inoculation was completed within 50 days after the outbreak of the second epidemic—that is, by the acme of the epidemic. The patients who were attacked a week after their inoculation are also excluded on account of their negative phase. Thus the local and personal conditions are fairly satisfactory, but the inoculation was too late, being begun only at the outbreak of the epidemic. The condition as to the time for inoculation, therefore, was not satisfactorily fulfilled. Figures in brackets show percentage.

	Non-inoculated.	Once inoculated.	Twice inoculated.
Number of persons	1,845	296	821
Number of influenza cases including catarrhal pneumonia	805	94	60
Percentage of persons	(43·6)	(31·7)	(7·3)
Deaths	55	3	1
Percentage of deaths to patients	(6·8)	(3·2)	(1·4)
Percentage of deaths to persons	(3·0)	(1·0)	(0·1)

In the above tables the morbidity for influenza is one-sixth as great among the twice inoculated persons as among the uninoculated, either in the roughly collected figures or in the more exact figures referring to families. This preventive rate is much greater than in our own test, probably owing to the lateness of inoculation. Thus one must expect a great deal of disagreement if the statistics are collected regardless of the three necessary conditions above proposed. The complications from catarrhal pneumonia are almost half as numerous among the twice inoculated patients as among the uninoculated. But when the rate of complicated cases is calculated on the total number, the decrease is about one-thirteenth among twice vaccinated persons as compared with those unvaccinated. The rates of these complications are similar to those in our own series. The deaths of patients whose cases were complicated with catarrhal pneumonia are very few among those twice vaccinated.

THE REACTION AFTER THE INOCULATION OF THE INFLUENZA VACCINE.

The inoculation was made after persons suffering from diabetes mellitus, nephritis, beri-beri and fever had been excluded, and also those in pregnancy. Almost all the inoculated had some local tenderness on pressure in the inoculated part and a slight feeling of illness for a day in the ordinary course. As a rule cessation from work was not required. In some sensitive people there was, in addition, a general reaction of fever and headache, but even this disappeared in two days.

In the Toyoda Factory 2282 persons were examined after the first inoculation, 5 were found to have ceased work for one day and one had fever over 37·5° C. In the same factory 2130 persons were examined after the second inoculation. Three ceased work for a day and 3 had fever. In the Kikuye factory after the first inoculation 18 out of 1468 persons ceased work but there were no cases of fever. In the Hidachi mining district 12 persons ceased work out of 9762 males and 4489 females after the first inoculation and 16 out of 6538 and 3154 after the second.

Thus the reaction after the inoculation with heated influenza vaccine is so slight that those inoculated need not be off work on account of bodily reaction except in a very few particular cases. As a general rule women have a somewhat stronger reaction than men.

CONCLUSION.

In our statistics, by the use of the heated influenza vaccine, the morbidity of those inoculated was reduced by a half as compared with the rate of those uninoculated. In other statistics, in which the inoculation was begun just at the commencement of the epidemic, and was finished by its acme, the morbidity among the inoculated persons, as compared with that among the uninoculated, was reduced to one-sixth by the use of the vaccine. The complications from catarrhal pneumonia were lessened in the case of inoculated patients to one-half, and deaths to one-seventh. The length of fever was less among the inoculated persons in proportion to the quantity of vaccine inoculated. The reaction after the inoculation was so slight that cessation from work was unnecessary.

Here I express my best thanks to Dr. M. Oshika, in the Toyoda factory, to Dr. M. Takami in the Kikuye factory, and to Dr. I. Kanasawa in the hospital of the Hidachi mining district. The first two kindly managed the inoculation and collected the statistics I wanted, and the last offered me the results of the test according to his plan.

REFERENCE.

Okawara, I., Tanaka, T., Watanabe, Y., Koyama, R., and Sato, T.—(1918) "On the Cause of Influenza that prevailed in 1918," *Kitasato Arch. Exper. Med.*, **2**, 235.

COMPARATIVE IMMUNITY TESTS WITH SALINE VACCINES AND LIPOVACCINES.

J. PRATT-JOHNSON, M.B., B.S.Lond., D.P.H.Oxon.

From the Bacteriological Department, Lister Institute, London.

Received for publication August 6th, 1921.

The presence of specific antibodies in the blood of immunised animals is of such general occurrence as to suggest that these substances are important factors in the mechanism of immunity. To what extent this is true is not known. In any case the antibody titre has been employed, perhaps too frequently, as a test of the immunity realised by the vaccination of man and of experimental laboratory animals. The type of immunity so arrived at may bear little or no relation to immunity as defined by ability of the vaccinated animals to withstand the dose or a multiple of the dose of the live organism or organismal protein which would otherwise kill the control unvaccinated animal.

The experiments here detailed were performed primarily for the purpose of studying the relative efficiency of lipovaccines and the ordinary saline vaccine suspensions. For this purpose it was planned to immunise various series of rabbits with vaccines prepared from a member of the Salmonella group possessing marked pathogenicity for the species (Tenbroeck's *Bacillus of Hog Cholera XII*), and, at a fixed interval after the close of immunisation, to test by the lethal dose method the degree of immunity, if any, attained. As we shall see, immunity as defined above was unfortunately not realised with any of the vaccines tried, and the experimental results here recorded bear simply on the lack of association of agglutinin development with genuine resistance to infection by the virulent live organism. In a subsequent paper (1921) some further and more successful attempts to obtain a solid immunity against the virulent strain in question are described.

PREPARATION OF LIPOVACCINES.

A lipovaccine may be defined as a suspension of killed bacteria in an oily vehicle. At first Le Moignic and Pinoy (1916) used mineral oils, which were subsequently replaced by vegetable oils such as olive oil, cotton-seed oil, almond oil, etc. In the French Navy lipovaccines have been employed on a large scale, and their preparation has been investigated by various workers, especially

Whitmore, Fennel and Petersen (1918), Olitsky (1918), and Rosenow and Osterberg (1919). It has been claimed that owing to the diminished toxicity of bacteria suspended in an oily vehicle lipovaccines may be administered in larger doses than saline vaccines, and that by virtue of their slower absorption a more lasting immunity is produced. The chief technical difficulty to be overcome in the preparation of lipovaccine is the removal of the watery envelope around the bacteria to be suspended. To this end various methods have been devised.

Method of Le Moignic.—The watery bacterial emulsion is centrifuged and the supernatant fluid removed. The semi-solid mass of bacteria is then again centrifuged and the remaining drops of watery fluid removed by means of a fine capillary pipette. A hydrophilic fluid of an oily nature, the composition of which is not published, is then allowed to remain in contact with the bacteria for some days, at the end of which time the bacteria, at first white, are penetrated by the oil and are stained reddish by the colouring matter in the oily fluid. The lipovaccine is sterilised by the combined action of "eugenol" and heating to 60° C. for thirty minutes.

Method of Whitmore, Fennel and Petersen.—The bacterial mass, after centrifugalisation, is dried *in vacuo* and finally broken up and incorporated in an oily vehicle by means of constant prolonged shaking in a special machine. The objections to this method are (1) the difficulty of breaking up the dried bacteria finely enough to obtain a uniform suspension, and (2) the question of ensuring the sterility of the vaccine, which is liable to contamination during the drying of the bacterial paste and its transference when dried to the special mixing machine. Further, it is doubtful if this method secures actual penetration of the oil into the bacteria.

Method of Olitsky.—After separation of the watery supernatant fluid by centrifugalisation, almond oil is added and a mixture made mechanically by vigorous shaking with glass beads. This method does not remove the water around the bacteria or secure penetration of the oil. The bacteria remain a greyish-white colour for many months and do not take up the colouring matter present in olive oil. Sterility is secured by heating to 60° C. for thirty minutes, the effectiveness of this low temperature in killing bacteria in an oily fluid demonstrating the presence of water in the lipovaccine.

Method of Rosenow and Osterberg.—The bacteria are collected by centrifugalisation and sterilised by heating or the addition of disinfectants. To the thick watery bacterial paste is then added cotton-seed oil containing 2 per cent. lanoline. The subsequent clearing process is hastened by the presence of the lanoline. The mixture is then heated under reduced pressure at 60° C., the contents being gently shaken until all bubbling has ceased. The water boils away, and the bacteria, which are at first of a greyish-white colour, become yellowish. Heating is continued until the opaque emulsion becomes almost clear.

An objection to this method is the employment of prolonged heating, which, if the brew be a large one, may extend up to three or four hours. The extent to which the antigenic value is affected would require investigation for each organism, and might be expected to vary also with the length of exposure to the heating process.

To ensure sterility of the final lipovaccine it is essential that a sterile bacterial emulsion be used in the first instance. Rosenow and Osterberg's method does secure penetration of the oil into the bacteria.

Eugenol method.—This process was suggested to the writer by a consideration of the analogous process of dehydration and clearing of pathological sections after staining by means of watery solutions. It will be remembered that the presence of mere traces of water produces clouding of the section owing to the incompatibility of water with xylol. Eugenol is a phenoloid substance present to a considerable proportion in commercial clove oil (about 80 per cent.), and has been adopted as an index of the purity of commercial clove oil. It behaves in every way similarly to clove oil but is preferable to it owing to the variable composition of the latter, which is liable to interfere with its clearing action especially when adulterated with alcohol.

The bacterial emulsion is first sterilised by heating to 60° C. for 30 minutes or 53° C. for 70 minutes. It is important to demonstrate its sterility because the subsequent process does not sterilise the thick emulsion. The emulsion is centrifugalised in a sterile tube plugged with cotton-wool and the supernatant fluid pipetted off. The deposit is again centrifugalised until it is packed down hard and the remaining drops of fluid removed with a sterile fine pipette. A few drops of absolute alcohol are then added to the centrifuge tube and the mass rubbed up with a small sterile glass pestle into a thick paste, which is gradually thinned down by adding more alcohol. The whole is again centrifuged and the alcohol removed by a fine pipette. A few drops of eugenol are then added and the sterile pestle again used to form a thick paste which is thinned down by the addition of more eugenol. In 15 to 20 minutes the bacteria are cleared up and penetrated by the eugenol. The lipovaccine is then made up by adding an estimated quantity of the eugenol suspension to sterile olive oil.

While this method has the advantage of simplicity and speedy preparation it is open to the possible objection that the alcohol washings are discarded. The presence of alcohol retards the subsequent clearing process by the eugenol, and also is liable to cause a precipitate when an alcohol-eugenol suspension is added to olive oil owing to the incompatibility of alcohol with olive oil. The antigenic value of the alcoholic washings which are discarded has not been investigated. The employment of alcohol is necessary to ensure the thorough breaking up and dehydration of the bacterial mass.

TECHNIQUE OF STERILITY TEST FOR LIPOVACCINES.

It has been shown that the sterilisation by means of heat of an oily suspension of living bacteria is an entirely different problem to the sterilisation of a watery suspension. The same temperature is required to destroy bacteria in oil as is necessary for bacteria in air. Under these circumstances in the preparation of lipovaccines it is essential that only dead bacteria be used for their preparation, as the high temperature necessary for the sterilisation of oils reduces or destroys the antigenic value of any suspended bacteria.

The addition of an oily fluid to a watery culture medium such as broth does not provide a satisfactory breaking up of the oil even by prolonged shaking. In view of the importance of definitely ascertaining the sterility of lipovaccines it appeared advisable to work out a technique which would secure a more satisfactory breaking up of the lipovaccine in the culture medium. If equal volumes of 1 per cent. sodium carbonate solution and olive oil be shaken up and then added to a large volume of distilled water a uniform milky fluid is produced. If this fluid be examined under a low power of the microscope it will be seen that the oil is split up into fine globular particles which are suspended in the fluid. If now this picture be compared with a preparation of a few drops of olive oil shaken up in ordinary broth the oil appears as large globular masses, only a small proportion of the oil having been split up into fine globular particles. After numerous experiments the following technique was adopted for testing the sterility of lipovaccines :

0·5 c.c. lipovaccine is pipetted by means of a 5 c.c. pipette into a sterile test-tube containing 2 c.c. of 1 per cent. sodium carbonate solution, and the oil rapidly emulsified by bubbling air through the mixture by means of the pipette. The whole is then sucked up into the pipette and immediately transferred to a 250 c.c. flask containing 50 c.c. of broth and well shaken. The short period of exposure of the oil to the alkali does not appear to be a matter of any practical importance, as *B. typhosus* was found to be still living after an exposure of 45 minutes to 1 per cent. sodium carbonate. It was not found necessary to correct the reaction of the broth to allow for the addition of alkali for the organisms investigated, but this adjustment could be readily worked out to obtain any desired reaction of the final emulsified oil-broth. The preliminary emulsification in this way provides a more efficient sterility test for oily preparations.

COMPARATIVE TESTS OF AGGLUTININ-DEVELOPMENT IN RABBITS IMMUNISED WITH SALINE VACCINES AND LIPOVACCINES.

A 24-hour-growth of the bacillus of Hog Cholera XII was emulsified in saline, shaken, filtered and counted by the dark-ground method. The emulsion was heated at 53° C. for 80 minutes, cooled down and made up to contain 0·5 per cent. carbolic acid. The strength of the final sterile emulsion was 270,000 million per c.c. This stock emulsion was used for the preparation of saline vaccines and lipovaccines.

Saline vaccines.—From this emulsion two saline vaccines were made, viz. :

(*a*) 5000 million per c.c.
(*b*) 10,000 million per c.c.

by diluting with carbolic saline.

Rosenow lipovaccine.—25 c.c. of the above sterile emulsion was centrifuged, the supernatant fluid removed, and sterile beads and 20 c.c. sterile neutralised olive oil containing 2 per cent. lanoline added. The large centrifuge tube was placed in a water-bath at 60° C. and connected up with a water-pump, a reduced pressure of 70-73 mm. of mercury being maintained. During the heating the tube was shaken by hand, the clearing process being completed in 1¼ hours ; 55 c.c. of sterile neutralised lanoline olive oil was added and the tube closed with a sterile rubber cork and the whole well shaken. After a satisfactory sterility test a lipovaccine was prepared for use of a strength of 15,000 million per c.c. by the addition of 5 c.c. of the emulsion to 25 c.c. lanoline-olive oil.

Eugenol lipovaccine.—4·4 c.c. of the sterile emulsion was placed in a sterile, plugged centrifuge tube. After centrifuging the supernatant fluid was removed by means of a pipette and the wet deposit again centrifuged, the last drops of fluid being removed by a fine pipette. The deposit was then rubbed up with a few drops of absolute alcohol into a thick paste by means of a sterile glass pestle, the paste being thinned down by the addition of further alcohol. After centrifuging, and pipetting off the alcohol, eugenol was added. At first a few drops were added and the mixture rubbed up into a paste by means of the glass pestle, the volume being subsequently made up to 2·4 c.c. After twenty minutes' treatment in eugenol a lipovaccine of 15,000 million per c.c. was prepared by dilution with sterile olive oil (0·93 c.c. added to 30 c.c. olive oil). The lipovaccine was found to be sterile.

Formalinised suspension.—A suspension was prepared from the original emulsion, and diluted down to a strength of 3000 million organisms per c.c. This suspension was sterilised by the addition of 0·1 per cent. formalin and was used throughout for all the agglutination tests.

Thirty-six rabbits were weighed and sorted out into three groups of similar weight distribution. Before inoculation the serum of all the animals was tested and found to produce no agglutination with the formalinised suspension except in one case where a weak reaction was obtained (1 in 40).

On November 2nd, 1920, the saline vaccine series received 5000 million organisms subcutaneously and the lipovaccine series (Rosenow vaccine 12 animals, eugenol vaccine 12 animals) 15,000 million organisms from their respective vaccines. On November 8th, 1920, the saline series received a further dose of 10,000 millions.

The temperature of all animals was taken night and morning up to November 11th, but except for a rise of 1–4° immediately following the first inoculation no special effects were observed. In no case was any marked local reaction obtained, and no induration or abscess occurred at the site of inoculation.

The serum of each of the thirty-six rabbits was tested on the ninth, thirteenth, sixteenth and twentieth days following the injection of the first dose of vaccine (November 2nd), and on each of these days, also, tests were made of the pooled sera of the twelve rabbits in each group.

The results of the pooled series are appended (Table I).

TABLE I.—*Agglutinin Titres of Rabbits after Inoculation with Saline Vaccines and Lipovaccines.*

Vaccine.	End-point titres on particular days after inoculation.			
	9th.	13th.	16th.	20th.
Saline	1280	2560	2560	2560
"Rosenow" lipovaccine	640	640	320	1280
"Eugenol" lipovaccine	80	160	160	320

I do not propose to detail the figures for the individual rabbits on the days in question, but it may be of interest to note the range of variation experienced in the three series (Table II).

TABLE II.—*Variation in Agglutinin Titre of Rabbits Inoculated with Saline Vaccines and Lipovaccines.*

	No. of animals yielding titres of							
	2560.	1280.	640.	320.	160.	80.	40.	20.
Saline vaccine	9	1	1	1	0	0	0	0
"Rosenow" lipovaccine	3	4	3	1	0	0	1	0
"Eugenol" lipovaccine	0	2	0	1	4	2	1	2

It will be noted that the saline vaccine yielded the best results, while the lipovaccines and especially the eugenol vaccine lagged far behind.

TEST FOR PROTECTION AGAINST THE LIVE ORGANISM.

On the twenty-second day (*i.e.* November 24th) 6 rabbits were taken from each group and given a test dose of 600 living organisms intravenously. The virulence of this organism for the rabbit had been tested previously on numerous occasions, deaths occurring within five to seven days with doses as low as 30 organisms. The actual minimal lethal dose was not ascertained, but from the preliminary experiments made it was clear that the test dose of 600 organisms represented not less than 20 minimal lethal doses. On the day of the test, however, 4 control animals were given 6000, 600, 600 and 60 organisms respectively. All died either on the fifth or on the sixth day.

The annexed table (Table III) shows the results with the vaccinated animals, none of whom survived the test dose. The presence of a high or a low agglutinin-content does not appear to have any influence on the date of death.

TABLE III.—*Date of Death of Infected Rabbits after Vaccination with Saline Vaccines and Lipovaccines.*

Saline vaccine.			Rosenow lipovaccine.			Eugenol lipovaccine.		
Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.
No. 22	5,120	Nov. 28	No. 35	2,560	Nov. 28	No. 46	40	Nov. 30
,, 23	80	,, 29	,, 36	2,560	,, 29	,, 47	20	,, 29
,, 24	2,560	,, 29	,, 37	1,280	,, 29	,, 48	160	,, 29
,, 25	1,280	,, 29	,, 38	320	,, 29	,, 50	80	,, 28
,, 26	640	,, 29	,, 40	320	,, 28	,, 51	160	,, 28
,, 27	10,240	,, 29	,, 41	640	,, 28	,, 52	1,280	,, 29

As it was clear that no protection had been established, it was decided to reinoculate the remaining six animals of each series. On November 30th each received 15,000 million organisms from their respective vaccines, the effect of this second dose being to raise very considerably the agglutinin-titres of the animals receiving the lipovaccine, almost to limits equalling those of the saline vaccine series.

On December 12th a test dose of 210 live organisms was given to each intravenously. At the same time five control animals received respectively 210, 210, 105, 56 and 28 live organisms. All five died between the fourth and seventh days, and as the annexed table (Table IV) shows, none of the vaccinated animals survived the test dose in spite of the high agglutinin-content of their sera.

Table IV.—*Date of Death of Infected Rabbits after Further Vaccination with Saline Vaccines and Lipovaccines.*

Saline vaccine.			Rosenow lipovaccine.			Eugenol lipovaccine.		
Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.
No. 28	640	Dec. 15	No. 39	2,560	Dec. 15	No. 49	320	Dec. 15
,, 29	10,240	,, 16	,, 42	5,120	,, 16	,, 53	5,120	,, 14
,, 30	5,120	,, 15	,, 43	2,560	,, 14	,, 54	2,560	,, 15
,, 31	10,240	,, 15	,, 44	2,560	,, 14	,, 55	2,560	,, 15
,, 32	10,240	,, 15	,, 45	5,120	,, 15	,, 56	320	,, 15
,, 33	2,560	,, 15	,, 34*	—	—	,, 57	640	,, 15

* This animal died in the course of immunisation.

CONCLUSIONS.

(1) Lipovaccines prepared and employed as above stimulate the production of agglutinins to a lesser degree than saline vaccines administered in the same doses.

(2) The presence of a high titre of agglutinins does not necessarily indicate the presence of an established immunity even against small doses of a highly virulent organism.

I here express my acknowledgments to the Governing Body of the Institute for facilities afforded me, and to Dr. Ledingham, Dr. Schütze and others for much helpful advice and criticism.

REFERENCES.

Johnson, J. P.—(1921) "Further Studies on the Production of Immunity in Rabbits against an Organism of High Virulence for the Species," *Brit. J. Exper. Pathol.*, **2**, 239.

Le Moignic and Pinoy.—(1916) "Les vaccines en émulsion dans les corps gras ou lipovaccines," *Compt. rend. Soc. biol.*, **79**, 201.

Le Moignic, E., and Sézary, A.—(1918) 'Nouvelle methode de vaccination antityphoidique,' Paris (Baillière et Fils).

Olitsky, P. K.—(1918) "An Experimental Study of Vaccination against Bacilli dysenteriæ," *J. Exper. Med.*, **28**, 69.

Rosenow, E. C., and Osterberg, A. E.—(1919) "A Method for the Preparation of Prophylactic and Autogenous Lipovaccines," *J. Amer. Med. Assoc.*, **73**, 87.

Whitmore, E. R., Fennel, E. A., and Petersen, W. F.—(1918) "An Experimental Investigation of Lipovaccines," *J. Amer. Med. Assoc.*, **70**, 427, 902.

FURTHER STUDIES ON THE PRODUCTION OF IMMUNITY IN RABBITS AGAINST AN ORGANISM OF HIGH VIRULENCE FOR THE SPECIES.

J. PRATT-JOHNSON, M.B., B.S.Lond., D.P.H.Oxon.

From the Bacteriological Department, Lister Institute, London.

Received for publication August 6th, 1921.

Previous attempts by the writer (1921) to protect rabbits against a virulent member of the Salmonella group (*B. choleræ suis* XII) by means of killed saline vaccines and lipovaccines having been unsuccessful, the following experiments were carried out with a view to testing the efficacy of immunisation with the live organisms of a related type of low virulence (*B. paratyphosus B*, type "Mutton"). No minimal lethal dose of the virulent organism had been determined, deaths having taken place with regularity after doses containing not more than 30 organisms. Tenbroeck (1918) has put on record certain experiments bearing on this question. He has shown that protection against lethal doses of "Hog cholera XII" can be secured by previous immunisation with live organisms of the "Mutton" but not of the "Schottmüller" type. It appeared therefore of prime importance to ascertain, if possible, the extent of this immunity in parallel experiment with the killed homologous and heterologous vaccines. The following vaccines were prepared:

(1) Killed saline vaccine from *B. choleræ suis* XII.
(2) Killed saline vaccine from *B. paratyphosus B*, type "Mutton."
(3) Living vaccine of *B. paratyphosus B*, type "Mutton."

The living "Mutton" vaccines were prepared and counted on each day of inoculation.

Immunisation.—Six animals were employed for each series. Three injections were given at intervals of seven days, the first injection (March 23rd) subcutaneously, and the second (March 30th) and third (April 6th) intravenously. The doses of the killed vaccines on these three dates were 2000 millions, 2000 millions and 6000 millions respectively, while the three doses of the live "Mutton" vaccines were 30 millions, 30 millions and 300 millions.

Agglutinin-development.—On the thirty-fourth day after the first injection (*i. e.* April 26th) the sera of all the animals were tested against both "Hog cholera XII" and "Mutton" emulsions. Table I shows the agglutination results in end-point dilutions.

TABLE I.

Vaccine group.	Rabbit serial No.	"Hog cholera XII."	"Mutton."
Killed "Hog cholera XII"	81	800	0
	82	100	0
	83	400	0
	84	200	0
	85	400	0
	86	1600	0
Killed "Mutton"	87	0	1600
	88	0	1600
	89	0	800
	90	0	1600
	91	0	1600
	92	0	800
Living "Mutton"	93	50	6400
	94	50	3200
	95	200	6400
	96	50	6400
	97	200	6400
	98	50	6400

It will be noted that only those animals immunised with the live "Mutton" vaccine developed agglutinins for both organisms. The group effect in this case is, however, associated with a remarkably high agglutinin-titre for the homologous organism.

Protection tests.—On April 27th, the day following the agglutination tests above detailed, the inoculated animals received test doses intravenously of the live virulent "Hog cholera XII." Two animals of each series received 200 organisms, two received 1000 organisms, and the remaining two 1,000,000 organisms, while three control animals received respectively 100, 1000 and 1,000,000 organisms. The results are given in Table II.

TABLE II.

Test dose of living "Hog cholera XII."	Killed "hog cholera" vaccine.		Killed "Mutton" vaccine.		Live "Mutton" vaccine.		Controls.	
	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.
200 organisms	81	May 4	87	May 2	93	*Survived*	101	May 2
	82	May 9	88	May 2	94	May 4	—	—
1000 organisms	83	May 3	89	*Survived*	95	*Survived*	102	May 2
	84	May 2	90	May 2	96	May 3	—	—
1,000,000 organisms	85	May 2	91	May 2	97	*Survived*	103	May 1
	86	May 2	92	May 2	98	May 4	—	—

It will be observed that of the six rabbits immunised with the killed "Hog cholera XII" none survived inoculation with the living culture, thus confirming previous results (1921).

Of the six animals immunised with the killed "Mutton" vaccine one survived, while of those which received the living "Mutton" organisms no less than three survived inoculation with living "Hog cholera XII." The series is a small one, and the immunity is not complete in any group, but the result demonstrates unequivocally the possibility of securing solid immunity against a highly virulent organism by vaccination with live members of a related type of low virulence. So far as this experiment teaches, no very obvious relation exists between survival, amount of test dose and date of death. With regard to antibody production, the chief point of interest is a possible association of immunity, not with the high agglutinin-titres following vaccination with the corresponding killed organism, but with the comparatively lowly titres which have developed in the serum as by-products of the action of a live avirulent but otherwise closely related organism.

In all cases the cause of death was verified as due to infection with "Hog cholera XII" by the recovery of this organism in pure culture from the heart-blood.

CONCLUSION.

Experiments are described which demonstrate the possibility of securing the immunity of rabbits against an organism of high virulence by immunisation with a live related organism avirulent for the species. In this respect Tenbroeck's observations are confirmed, and it is further shown that killed vaccines prepared either from the homologous or from a related organism fail to give solid immunity against the live virulent strain.

REFERENCES.

Johnson, J. P.—(1921) "Comparative Immunity Tests with Saline Vaccines and Lipovaccines," *Brit. J. Exper. Pathol.*, **2**, 232.

Tenbroeck, C.—(1918) "A Study of Paratyphoid Bacilli isolated from Cases of Hog Cholera," *J. Exper. Med.*, **28**, 759.

THE pH RANGE FOR STAPHYLOCOCCI.

I. WALKER HALL AND A. D. FRASER.

From the Pathological Department of the Bristol University.

Received for publication June 20th, 1921.

In the estimations of the relation of hydrogen ion concentrations to the several periods of bacterial life the staphylococcal group has been but rarely employed. It was therefore deemed of interest to examine a series of strains derived from such varying sources as boils, abscesses, sycosis, sinusitis, etc., and from the nose, mouth, skin, genital tract and urine. To the simple determinations of the limiting pH range for these organisms there was added an inquiry as to the association of virulence, the acid or alkali resisting properties, and the preservation of such characters during successive cultivations at pH ranges near the lethal point. It was hoped thereby to discover some means of altering the antigenic capacities of the coccus or its secretions, so as to evolve a more satisfactory means of dealing with the residue of chronic skin and sinus infections which evade the ordinary means of treatment.

METHODS.

A large batch of lait-proto broth (Hall, 1918) was prepared from pure casein, and standardised as to its amino-acid and pH content. The medium was sugar-free, and contained not more than 1 per cent. of NaCl after the final adjustments. This was designed to eliminate the interaction of simultaneous acid and alkaline fermentations, and to provide against the effect of considerable acidity produced during growth. From this standard broth a number of pH ranges were prepared.

Although the changes in pH ranges during growth were determined in several instances, chief attention was devoted to the behaviour of the cocci in an initial hydrogen ion concentration.

The *variations* connected with the use of cultures of differing *ages* was met by the daily transfer of each strain for several days prior to the experiment, and the experimental seeding was made from a four-hour culture in the plain lait-proto broth.

The *amount of inoculum* employed was a standard loopful in all instances, and this was sufficiently large to ensure that the *period of logarithmic increase* fell between four and five hours.

The intensity of growth was determined by the opacity method in this instance, as the period of logarithmic increase formed the subject of another inquiry. The opacity standards were made up in the same broth, with the usual quantities of barium chloride and sulphuric acid. It was found at an early stage that the density of staphylococcal growths is due partly to ether-soluble substances, and that it was necessary to remove them by extraction before the readings were taken. With this precaution the method possesses several advantages, and was found for the specific purpose in view to yield more regular results than bacterial counts. For determination during the maximal periods of growth, however, the use of enumeration methods, though possessing inherent fallacies, was made use of with necessary controls. In this inquiry the readings were taken after twenty-four hours and seven days.

pH INITIAL RANGE FOR STAPHYLOCOCCI DERIVED FROM DIFFERENT SOURCES.

In order to ascertain the pH range for staphylococci and how far it varied for individual strains, a series of broth flasks from the same batch were adjusted with N/HCl or N/NaOH to pH 2·6, 5·0, 7·6, 8 and 10. The seedings were taken from the third subculture of each strain.

Growth after twenty-four hours.—It will be noted from Table I that during the first twenty-four hours certain strains failed to grow in an acidity below pH 5·0, while others derived from a similar type of lesion grew quite easily in pH 2·6. On the other hand, a few of the strains were inhibited by alkalinity beyond pH 8, although the majority grew quite easily at pH 10.

TABLE I.—*The Limiting pH Ranges for Staphylococcal Strains.*

	Source of organism.	Number of strains.	pH concentrations.							
			Twenty-four hours.				Seven days.			
			2·6.	5·0.	8.	10.	2·6.	5·0	8.	10.
S. aureus	Boils	11	8	3	1	10	10	1	1	10
,,	Abscess	4	2	2	—	1	2	2	—	1
,,	Sycosis	1	—	1	—	1	1	—	—	1
,,	Spinal fluid	1	1	—	—	1	1	—	—	1
S. albus	Sputum	1	—	1	—	1	—	1	—	1
,,	Antrum	4	3	1	—	1	3	1	—	1
,,	Nose	1	1	—	—	1	1	—	—	1
,,	Skin	1	—	—	—	1	1	—	—	1
,,	Throat	2	1	1	1	1	1	1	—	2
,,	Urine	2	1	1	—	2	2	—	1	1
,,	Uterus	1	1	—	—	1	1	—	—	1

Growth after seven days.—When the results of the seven days' incubations are considered, it has to be remembered that Kendall and Farmer (1912, 1913) have shown that in the absence of dextrose, or any other utilisable sugar, the carbohydrate radical of the protein molecule is readily attacked by staphylococci.

Hence in plain broth the same workers found, using neutral red as an indicator, that staphylococci produced acid up to 1 per 100 c.c. for five days, and then alkali to 1 per 100 c.c. for seven days, and 2 per 100 c.c. on the eighth day.

Two strains derived from "boils," one from sycosis and another from urine, all exhibited growth in a higher pH range after seven days, although they failed to do so at an earlier stage. This suggests that our pH 2·6 was simply a delaying and not a lethal range, and that the cocci attained a growth sufficient for registration during the two latter days. We have not burdened the table with the records of the intensity of the growths. A standard opacity was required for the placing of each strain during the readings.

Thus there are variations in the pH limiting range of staphylococci. The differences appear to be inherent in the individual strains, and do not exhibit any relation to the saprophytic or pathogenic characters of the organism. After the first twenty-four hours the cocci commence to grow more easily at the extreme limits.

CHANGES IN SUGAR-FREE MEDIA.

The general action of staphylococci in sugar-free media has already been noted, and the appearance of the secondary fermentation has been observed to coincide with an increase in the amount of growth in the cultures of optimum pH, and an indication of growth in the zones of limiting pH range. It now remains to follow out these changes in the several concentrations and to note how far they are influenced by the respective conditions.

Considering the results as a whole, the extent of alkaline phase is more early and intense the more acid or more alkaline the medium, and less so at the optimal concentration.

For this purpose the organisms were subcultivated several days in the same pH range of broth, and thus finally grown for seven days at 37° C. The changes which follow are, perhaps, more clearly shown when the control and the inoculated media are adjusted to pH 7·5, and the quantities of alkali or acid required are expressed in terms per litre. A single example will suffice to demonstrate the general findings, although the various strains differ also slightly in this characteristic.

TABLE II.—*Alkaline Reactions in Seven Days' Staphylococcal Growths at 37° C. Strain No. 13 from "Boils."*

	Amount of acid or alkali in c.c. per litre required to adjust media to pH 7·5.	
	Medium only.	Medium *plus* staphylococci.
pH 2·6	19·1	14·1
pH 4·0	6·0	4·0
pH 5·0	2·0	1·0
pH **7·5**	**0·2**	**1·0**
pH 9·0	8·0	11·0
pH 10·0	12·0	15·0

ALKALI OR ACID RESISTANT STRAINS.

The variations exhibited by the different strains suggested the possibility of training the organisms to proliferate under higher or lower pH concentrations.

Accordingly a number of strains which grew freely in pH 2·6 broth were subcultured daily for twenty-eight days in pH 2·6 media. Standard loopfuls were transferred on the 1st, 3rd, 7th, 14th, 21st and 26th days into all the pH ranges. Growth could not be obtained in broths more acid than pH 2·6 or more alkaline than pH 10.

A further attempt to produce an experimental adaptation towards the acid or alkaline side was made by allowing the cocci to remain in the pH 2·6 and 10 broths for seven days before transferring them to new broths., It failed equally with the 7th, 14th, 21st and 28th day subcultures. Beyond the limits of pH 2·6 and 10 it seemed impossible to go, so long as the same broth was employed. It was observed, however, that after the 14th day the organisms grew more readily and reached the standard opacity at an earlier hour than hitherto.

CHANGES IN MEDIA CONTAINING ACIDS OTHER THAN HCl.

As it is sometimes necessary to bring media to pH 7·6 by the addition of minute quantities of N/10 HCl, it was considered worth while to test the capacity of the various strains in the presence of other acids. Accordingly a quantity of lait-proto media was prepared and adjusted to 20 amino-acid content, made neutral to phenolpthalein with N/1 NaOH, and then readjusted to the several pH contents with various normal acid solutions. Those selected were nitric, sulphuric, phosphoric, acetic, citric, tartaric, oxalic, lactic, butyric and salicylic. The full details will be given in a subsequent paper upon the action of the diluted acids upon other organisms. The main results with staphylococci indicated a limiting of the pH range by the use of salicylic, sulphuric and tartaric acids. The strains which failed to grow easily in HCl pH 2·6 media failed also in the other solutions. In the pH 5·0 series the salicylic broths were the only set which did not yield good growths. The remainder, in contrast with a retarded proliferation in the 2·6 group, exhibited good opacity within 24 hours. In both groups the nitric acid broths——roughly a dilution of 1 in 50, and 1 in 90 N/10 HNO_3—exhibited a distinct acceleration, resulting in a striking growth at 7 hours and a maximum growth at 24 hours.

Barnes (1921), working with milk as a medium for staphylococcal growth, considers that staphylococci may be divided into sub-groups according to the extent of their enzymic action upon milk proteins, and concludes that there is some relation between the power of liquefying gelatine and that of curdling milk. Similarly we have reason to regard the sub-grouping of these organisms from the standpoint of their acid or alkali-resisting capacities. It may be that our progressing correlation of these features with hæmotoxin production may lend further aid in the recognition of differing types. At all events, there is a sufficiency of data to prompt a closer examination of the antigenic powers of emulsions prepared for therapeutic measures.

SUMMARY.

The general pH range for the staphylococcal group extends from pH 2·6 to pH 10. But individual or group variations occur.

These variations do not exhibit any relation to saprophytic or pathogenic characters.

They do not admit of much experimental adaptation.

The capacity for alkali production in sugar-free media is more evident in lower than higher pH concentrations. By substitution of various acids in the acid series a limiting of the pH range has been produced on the one hand and a definite acceleration of growth on the other, nitric acid being an outstanding adjuvant.

REFERENCES.

Barnes, W. H.—(1921) "The Activity of Staphylococci in Milk," *J. Infect. Dis.*, **28**, 259.

Hall, I. W.—(1918) "On the Amino-acid Content of Nutrient Media," *Brit. Med. J.*, **2**, 398.

Kendall, A. I., and Farmer, C. J.—(1912) "Studies in Bacterial Metabolism," *J. Biol. Chem.*, **12**, 216.—(1913) *ibid.*, **13**, 68.

The expenses of this work were met by grants from the Medical Research Council and the University of Bristol Colston Research Society. We wish to acknowledge the painstaking assistance we have received from Mr. F. Nicholls.

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

THE RELATIONSHIP OF BLOOD-GROUPS TO DISEASE.*

J. ARTHUR BUCHANAN, M.D.,
Fellow in Medicine,
AND
EDITH T. HIGLEY, B.S.,
Fellow in Clinical Pathology.

From the Mayo Foundation, Rochester, Minnesota.

Forwarded for publication August 16th, 1921.

THE possibility of a fixed relationship of "blood-group" to a particular disease or diseases has received practically no consideration. A fundamental property of blood was recognised first by Landois Eulenburg (1866), when he demonstrated that the serum of one animal may have the property of destroying the red cells of another animal of the same species. This property is now known as isohæmolysis, and is of prime importance in the therapeutic application of our knowledge of blood grouping. On isohæmolysis certain poor results of transfusion depend; thus it is necessary to make a proper blood-grouping.

The salient feature on which the actual grouping of blood rests was discovered by Ehrlich (1900). He observed the agglutination of erythrocytes by serum from animals of the same species. Ehrlich called this property iso-agglutination. At a much earlier period Jones (1853) and, later, Paget (1871) observed a peculiar tendency of blood from persons with certain acute infections to undergo a rapid agglutination (rouleau formation) when poured on a glass plate. Shattock (1900), stimulated by the work of Landois and

---

* This investigation was made in the Department of Clinical Laboratories of the Mayo Clinic under the direction of Dr. A. H. Sanford.

Ehrlich, and interested by the observations of Jones and Paget, was the first to observe iso-agglutination in man. He mixed the serum of a patient with pneumonia with the erythrocytes of a normal person. He observed prompt agglutination of the red cells. He later made the same test with the serum of a patient with acute rheumatism and the erythrocytes of a patient with leukæmia. The same phenomenon occurred. He watched the rheumatic patient through convalescence; the peculiarity persisted.

Landsteiner (1901), influenced by the work of Shattock, began matching the blood of healthy persons. He was able to classify all persons, because of the properties of their blood, into three groups. The groups described by Landsteiner are now known as Groups II, III and IV. In a later study Jansky (1907) discovered a fourth group, now known as Group I. The same observation was made independently by Moss (1909). A difference in their groupings was that Jansky considered Group I the common group and Group IV the uncommon group. Moss classified his groups in the reverse order. At the Mayo Clinic the Moss classification is used, although the priority of Jansky is recognised.

Hektoen (1907) demonstrated that disease fails to modify iso-agglutination in man. Bond (1920) has recently called attention to the frequency of auto-agglutination in the presence of a positive Wassermann reaction, focal infection, pneumonia, empyema, typhoid fever, carcinoma of the uterus with secondary infection, and in a number of cases of infective endocarditis. Such phenomena have not been observed in the Mayo Clinic.

The Mendelian transmission with regard to blood-grouping was studied by von Dungern and Hirschfeld (1911), who concluded that the groups were transmissible according to the Mendelian laws. Their study consisted of seventy-one families, and their investigations were carried through only two generations. Ottenberg and Epstein (1908) also presented data in accord with the findings of von Dungern and Hirschfeld. This phase of the problem needs further investigation, because transmission of more than one character requires study through more than two generations to prove the fulfilment of the Mendelian laws. This fact becomes evident when families are grouped for the purpose of securing a donor, and often groups are isolated that are not represented in either parent.

The data of the Mendelian transmission were accepted by Alexander (1921), who was led to make a series of studies on certain patients in order to find if there is linkage with a factor on which certain blood diseases depend. He studied particularly malignancy, and concluded "that while persons belonging to all four groups are liable to malignant disease, those in Groups I and III appear to be peculiarly susceptible, and the clinical type of disease is generally speaking more malignant." Alexander failed to consider the influence of nationality in his series.

The data presented in our study is a review of the blood-groups found in patients in the Mayo Clinic from January 1st, 1917, to May 1st, 1921. The relationship between blood-grouping and disease is shown in Tables 1 to 17, but only when a considerable number of cases were available have the figures been carried out into percentages. Percentages based on a small number of cases are quite often misleading.

For the sake of comparison the original percentages of Moss and of Hirschfeld and Hirschfeld (1919) on nationality and blood-grouping are discussed.

Original Percentages of Moss.

Group I	10 per cent.
,, II	40 ,,
,, III	7 ,,
,, IV	43 ,,

Abbreviated Table of Hirschfeld and Hirschfeld on Blood-grouping and Nationality.

	Group I per cent.	Group II per cent.	Group III per cent.	Group IV per cent.
English	3·0	43·4	7·2	46·4
French	3·0	42·6	11·2	43·2
Italians	3·8	38·0	11·0	47·2
Germans	5·0	43·0	12·0	40·0
Austrians	8·0	40·0	10·0	42·0
Serbs	4·6	41·8	15·6	38·0
Greeks	4·0	41·6	16·2	38·2
Bulgarians	6·2	40·6	14·2	39·0
Arabs	5·0	32·4	19·0	43·6
Turks	6·6	38·0	18·6	36·8
Russians	6·3	31·2	21·8	40·7

TABLE 1.—292 *Cases of Carcinoma.*

	Group I.	Group II.	Group III.	Group IV.
United States	6	87	15	99
Russia	1	5	—	1
Scandinavia	2	11	4	10
Germany	2	11	1	11
England	—	2	1	16
Bohemia	—	2	—	2
Poland	—	1	—	—
Austria	—	—	1	—
France	—	—	—	1
	11 (3·76 %)	119 (40·75 %)	22 (7·53 %)	140 (47·94 %)

The data in Table 1 contain nothing to show a relationship of the factor or factors responsible for the blood-group to carcinoma. The percentages for Groups I and III are entirely out of accord with those of Alexander.

Table 2.—457 *Cases of Pernicious Anæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	17	147	31	144
England	1	16	6	21
Russia	1	3	—	1
Scandinavia	1	19	6	17
Germany	—	15	3	4
Belgium	—	2	—	1
Italy	—	—	—	1
	20 (4·37 %)	202 (44·20 %)	46 (10·06 %)	189 (41·35 %)

The percentages in Table 2, although at slight variance with the original percentages of Moss, demonstrate that the causal factor in pernicious anæmia has no relation to blood-grouping.

Table 3.—11 *Cases of Acute Lymphatic Leukæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	3	3	4
Germany	—	—	—	1
	—	3	3	5

Table 4.—16 *Cases of Chronic Lymphatic Leukæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	3	—	9
England	—	1	—	—
Scandinavia	—	1	—	—
Russia	—	—	1	—
Germany	—	—	1	—
	—	5	2	9

Table 5.—47 *Cases of Myelogenous Leukæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	1	12	6	15
England	—	2	2	3
Russia	—	—	1	—
Scandinavia	—	—	2	1
Germany	—	—	—	2
	1	14	11	21

TABLE 6.—41 *Cases of Splenic Anæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	14	1	19
Scandinavia	—	1	—	—
Russia	—	1	—	1
Austria	—	1	—	—
England	—	—	—	2
Greece	—	—	—	1
	—	17	1	23

TABLE 7.—20 *Cases of Hæmophilia and* 20 *Cases of Purpura.*

	Group I.	Group II.	Group III.	Group IV.
United States	1	13	3	18
Russia	—	3	1	—
Scandinavia	—	—	—	1
	1	16	4	19

TABLE 8.—62 *Cases of Acute and Chronic Kidney Disease.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	17	3	22
England	—	2	1	7
Austria	—	2	—	—
France	—	—	—	1
Germany	—	—	—	3
Poland	—	—	—	1
Scandinavia	—	—	—	3
	—	21	4	37

TABLE 9.—20 *Cases of Cardiac, Valvular and Myocardial Disease.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	6	2	10
England	—	1	—	—
Scandinavia	—	—	1	—
	—	7	3	10

TABLE 10.—37 *Cases of Diseases of the Thyroid.* (15 *Cases of Exophthalmic Goitre,* 19 *Cases of Adenoma without Hyperthyroidism,* 2 *Cases of Myxœdema, and* 1 *Case of Colloid Goitre.*)

	Group I.	Group II.	Group III.	Group IV.
United States	—	9	1	19
England	—	2	1	2
Scandinavia	—	1	—	1
Germany	—	—	—	1
	—	12	2	23

TABLE 11.—65 *Cases of Tumours, Benign and Malignant, other than Cancer.*

	Group I.	Group II.	Group III.	Group IV.
United States	1	15	6	22
Italy	—	1	—	2
England	—	1	—	3
Scandinavia	—	3	1	2
Germany	—	1	1	3
Austria	—	—	2	1
	1	21	10	33

The data in Tables 3 to 11, although small, are sufficient to justify the suspicion that there is no linkage.

TABLE 12.—134 *Cases of Calculi.* (130 *Cases of Cholelithiasis,* 2 *Cases of Nephrolithiasis, and* 2 *Cases of Vesicolithiasis.*)

	Group I.	Group II.	Group III.	Group IV.
United States	4	39	9	48
Scandinavia	—	8	1	—
England	—	2	—	10
Germany	—	4	1	1
Bohemia	—	1	—	1
Russia	—	1	1	1
Switzerland	—	—	—	1
France	—	—	—	1
	4 (2·97 %)	55 (41·04 %)	12 (8·95 %)	63 (47·01 %)

TABLE 13.—137 *Cases of Fibroid Uterus.*

	Group I.	Group II.	Group III.	Group IV.
United States	3	52	11	45
Scandinavia	3	2	—	2
England	—	7	2	5
Switzerland	—	1	—	—
Germany	—	2	—	1
Belgium	—	—	—	1
	6 (4·37 %)	64 (46·71 %)	13 (9·48 %)	54 (39·41 %)

Table 14.—194 *Cases of Secondary Anæmia from all Causes.*

	Group I.	Group II.	Group III.	Group IV.
United States	8	62	16	56
England	1	10	4	15
Germany	—	4	1	5
Holland	—	1	—	—
Scandinavia	—	2	—	4
Bohemia	—	1	—	—
Russia	—	2	—	1
Austria	—	—	—	1
	9	82	21	82
	(4·63 %)	(42·26 %)	(10·72 %)	(42·26 %)

Table 15.—176 *Cases of Acute and Chronic Gall-Bladder Disease.*

	Group I.	Group II.	Group III.	Group IV.
United States	5	41	10	69
Germany	1	4	1	4
Bohemia	—	1	—	1
Austria	—	1	—	—
England	—	7	—	19
Scandinavia	—	6	1	—
Holland	—	1	—	—
Switzerland	—	—	—	1
Russia	—	1	1	1
	6	62	13	95
	(3·40 %)	(35·39 %)	(7·38 %)	(53·97 %)

There is a slight diminution in the percentages for Group II as shown in Table 15 with a considerable increase in Group IV. This, however, can have nothing to do with heredity.

Table 16.—172 *Cases of Chronic Ulcers.* (42 *Cases of Gastric Ulcer,* 102 *Cases of Duodenal Ulcer,* 4 *Cases of Gastro-jejunal Ulcer,* 15 *Cases of Chronic Ulcerative Colitis, and* 8 *Miscellaneous Cases.*)

	Group I.	Group II.	Group III.	Group IV.
United States	5	44	3	76
England	2	2	3	15
Scandinavia	—	7	—	5
Germany	—	2	—	3
Austria	—	—	2	—
Bulgaria	—	—	—	1
Russia	—	—	—	1
Honduras	—	—	—	1
	7	55	8	102
	(4·07 %)	(31·97 %)	(4·65 %)	(59·30 %)

TABLE 17.—173 *Cases of Jaundice, all Causes.*

	Group I.	Group II.	Group III.	Group IV.
United States	5	63	12	46
Germany	1	7	2	2
England	—	6	1	14
Italy	—	1	—	—
Scandinavia	—	6	3	—
Belgium	—	—	1	—
Austria	—	—	1	—
Bohemia	—	—	—	1
Switzerland	—	—	—	1
	6 (4·04 %)	83 (47·97 %)	20 (11·56 %)	64 (36·99 %)

TABLE 18.—372 *Miscellaneous Cases. (Group of Patients with Various Non-Malignant Diseases.)*

Group I.	Group II.	Group III.	Group IV.
19	150	26	177
(5·10 %)	(40·32 %)	(6·96 %)	(47·58 %)

TABLE 19.—2446 *Cases of Combined Diseases.*

Group I.	Group II.	Group III.	Group IV.
91	988	221	1146
(3·31 %)	(40·45 %)	(9·09 %)	(46·85 %)

The analysis of the blood-groups in 2446 patients (Table 19) gives percentages that are quite different from those originally found by Moss. The variation is explainable by nationality, and in some instances by the small number of patients considered in certain series. The difference is noticeable in all the tabulations, and for that reason it is well to consider the figures of Moss as only approximate, since they vary within certain limits in each group in each series. Sanford (1920), in a statistical study of about 3000 patients and donors, found 4·5 per cent. in Group I, 42·3 per cent. in Group II, 8·7 per cent. in Group III, and 44·5 per cent. in Group IV. Since then Culpepper and Ableson (1921), in an analysis of 5000 blood-groups, found that there were 5·18 per cent. in Group I, 35·06 per cent. in Group II, 14·28 per cent. in Group III, and 44·47 per cent. in Group IV.

CONCLUSIONS.

(1) There is no relationship between blood-groups and malignancy as suggested by Alexander.

(2) There is no relationship between blood-groups and any disease in which sufficient data are available to justify a conclusion.

(3) The percentages originally presented by Moss are approximate and capable of considerable variation, without special significance.

(4) Nationality should be taken into consideration in the presentation of statistical studies of blood-grouping.

REFERENCES.

Alexander, W. (1921).—"An Inquiry into the Distribution of the Blood-Groups in Patients suffering from 'Malignant Disease,'" *Brit. J. Exper. Pathol.*, **2**, 66.

Bond, C. J. (1920).—"On Auto-hæmagglutination: A Contribution to the Physiology and Pathology of the Blood," *Brit. Med. J.*, **2**, 925, 973.

Culpepper, W. L., and Ableson, Marjorie (1921).—"Report on 5000 Bloods typed using Moss's Grouping," *J. Lab. and Clin. Med.*, **6**, 276.

von Dungern, E., and Hirschfeld, L. (1911).—"Ueber die Giftigkeit des Blutes nach der Injektion protoplasmatischer Substanzen und während der Schwangerschaft und über passive Allergie gegenüber Hodensubstanzen," *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, **8** (Orig.), 332.

Ehrlich, P., and Morgenroth, J. (1900).—"Ueber Hæmolysine," *Berl. klin. Wchnschr.*, **37**, 453.

Eulenburg, A., and Landois, L. (1866).—'Die Transfusion des Blutes,' Berlin (Hirschwald).

Hektoen, L. (1907).—"Iso-agglutination of Human Corpuscles with respect to Demonstration of Opsonic Index and to Transfusion of Blood," *J. Amer. Med. Assoc.*, **48**, 1739.

Hirschfeld, L., and Hirschfeld, H. (1919).—"Serological Differences between the Blood of Different Races: The Result of Researches on the Macedonian Front," *Lancet*, **2**, 675.

Jansky, J. (1907).—"Hæmatologische Studien bei Psychotikern," ref. *Jahresb. f. Neurol. u. Psychiat.*, **11**, 1092.

Jones, T. W. (1853).—"Observations on the State of the Blood and the Blood-vessels in Inflammation," *Tr. R. Med.-Chir. Soc. Lond.*, **36**, 391.

Landsteiner, K. (1901).—"Ueber Agglutinationserscheinungen normalen menschlichen Blutes," *Wien. klin. Wchnschr.*, **14**, 1132.

Moss, W. L. (1909).—"Studies on Iso-agglutinins and Isohæmolysis," *Tr. Assoc. Amer. Phys.*, **24**, 419.

Ottenberg, R., and Epstein, S. (1908).—*Tr. New York Pathol. Soc.*, **8**, 117.

Paget, J. (1871).—'Lectures on Surgical Pathology,' 3rd Ed. Philadelphia (Lindsay & Blakiston), p. 235.

Sanford, A. H. (1920).—"Blood Transfusion: Indications for its Use, Methods of Selecting Donors, and a Brief Consideration of Technic," *Med. Clin. N. Amer.*, **3**, 801.

Shattock, S. G. (1900).—"Chromocyte Clumping in Acute Pneumonia and certain other Diseases, and the Significance of the Buffy Coat in the Shed Blood," *J. Path. and Bacteriol.*, **6**, 303.

DYSENTERY IMMUNISATION IN RABBITS BY THE ORAL AND SUBCUTANEOUS METHODS.

S. KANAI, M.D.,

Tokyó.

From the Bacteriological Department, Lister Institute, London.

Received for publication November 9th, 1921.

THE possibility of immunisation *per os* was first demonstrated by Ehrlich (1891), who succeeded in eliciting the development of anti-ricin in rabbits by feeding them with ricin. This question was left more or less in abeyance until 1905, when Zeitlin (1905) showed that rabbits, to which a killed suspension of *B. dysenteriæ* (Shiga) had been administered orally, developed agglutinins for that organism in their serum. In view of these findings he suggested that the oral route should be employed in the prophylactic immunisation of man against such acute intestinal diseases as cholera, dysentery and enteric. Working along these lines Hida and Toyoda (1907) demonstrated the production of antibodies in guinea-pigs by the administration *per os* of dysentery bacilli (type Shiga) previously digested with pepsin and trypsin. The ingestion of dysentery bacilli, which had merely been killed by heating to 60° C., was, according to these workers, unable to give rise to any antibody response, but Shiga (1908) claims to have immunised rabbits against this same organism by the oral administration of heat-killed vaccines.

More recently Besredka (1919) brings forward experimental evidence in support of oral immunisation. He claims that a solid immunity, as tested by intravenous injection of the live organism, can be produced in rabbits against *B. dysenteriæ* (Shiga), *B. paratyphosus B.* and *B. typhosus* by the introduction *per os* of killed vaccines. Rabbits immunised in this manner were able to support the intravenous inoculation of otherwise lethal doses of living organisms. This method of immunisation was found by him to be more efficacious in the cases of *B. paratyphosus* and *B. typhosus* if the vaccine was mixed with a certain quantity of sterile bile. From his observations Besredka concluded that the intestinal mucosa forms the first line of defence against invasion by these organisms—or the toxic products of these organisms—which give rise to acute intestinal conditions. This contention is supported by the observation that the first ingestion of antigen gave rise to the appearance of antibody in the blood, whereas the subsequent ones did not; in fact, at that period where he was able to demonstrate immunity in his animals, the agglutinins had practically disappeared. This immunity he therefore considers to be mainly local—an increased resistance on the part of the cells of the intestinal mucous membrane to the passage of the infecting organism or of its toxin—the damaged cells which are cast off in the catarrhal condition set up by the infection being replaced by new ones possessed of this acquired property. Besredka looks upon this initial catarrhal stage as essential to the establishment of a satisfactory local immunity, and for this reason bile is administered with the vaccine.

In appraising the practical value of any method of prophylactic immunisation we should consider the following points:

(I) The degree of immunity obtained.
(II) The intensity of reaction, local and general, produced by the vaccine.
(III) The simplicity of administration.

It is generally recognised that the local and general reactions following the subcutaneous inoculation of Shiga vaccines in all but low doses are often intense. In view, therefore, of Besredka's finding that a solid immunity may result from the oral administration of the vaccine, it was decided to carry out experiments in rabbits with the object of determining the degree of such resistance to *B. dysenteriæ* (Shiga), and to compare it with the immunity resulting from the usual subcutaneous inoculation.

SCHEME OF RESEARCH.

First Series of Experiments.

Two strains of *B. dysenteriæ* (Shiga) were employed in this investigation. One came from the National Collection of Type Cultures (No. 151), and for the other I am indebted to the kindness of Dr. Besredka. Both these strains were typical, culturally and serologically. The work reported in this communication was carried out with strain No. 151 practically entirely.

Ingestion of vaccines.—The 24-hour growth on agar obtained from one Roux bottle was suspended in 20 c.c. of sterile normal saline and heated for one hour at 60° C. Measured quantities of this suspension were mixed with bran and fed to rabbits which had fasted for 24 hours.

Antibody production (*agglutinins*).—Test bleedings were made before, and at intervals during the immunisation. The titre of antibody was determined by means of the agglutination reaction, results being read after four hours' incubation at 37° C. and again after standing at room temperature for twenty-four hours.

Degree of immunity produced.—This was determined by the intravenous inoculation of lethal doses of living *B. dysenteriæ* (Shiga), the virulence of which for the rabbit had been previously determined.

EXPERIMENTAL DATA.

The Virulence of Strain No. 151.

A saline suspension of a 24-hour agar slope culture was inoculated intravenously in a series of four rabbits, the dose varying from 50×10^6 to 500×10^6. The results are recorded in Table I:

TABLE I.

No. of rabbit.	Weight.	Number of bacilli inoculated.	Result.
17	1740 grm.	500×10^6	Died 2 days after injection.
18	1535 ,,	250×10^6	,, 8 ,, ,, ,,
19	1700 ,,	100×10^6	Survived.
20	1450 ,,	50×10^6	Survived.

Post-mortem Findings.

No. 17.—Small intestine congested and mucous membrane œdematous. The organism was recovered from the intestinal contents but not from the spleen.

No. 18.—Small intestine showed the same changes as in Rabbit 17. No hæmorrhages. Large intestine and appendix appear normal. Cultures from the heart-blood, gall-bladder, spleen and intestinal contents proved sterile.

From these experiments it would appear that the M.L.D. of this strain was about 500×10^6. In later experiments this dose was sometimes found insufficient to kill medium-sized rabbits, and accordingly 1000×10^6 was adopted as the test dose.

FIRST SERIES OF EXPERIMENTS.

Effect of Oral Immunisation.

Eight rabbits were immunised, as follows:

Nos. 5 and 6 received $\frac{1}{2}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 7 and 8 received $\frac{1}{5}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 9 and 10 received $\frac{1}{10}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 11 and 12 received $\frac{1}{5}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

In the two latter (Nos. 11 and 12) the ingestion was preceded by the oral administration of 8·0 c.c. of ox bile. Of these eight rabbits two died, No. 5 on the fifteenth day after ingestion and No. 8 on the fourteenth day. No obvious cause for death was found. The remaining six rabbits were divided into two groups. Group 1 (Nos. 6, 7 and 9) were inoculated with 500×10^6 living bacilli intravenously on the nineteenth day after the ingestion of the killed dysentery bacilli. Group 2 (Nos. 10, 11 and 12) received 1000×10^6 living bacilli twenty-six days after the immunising dose. The result of the experiment is given below (Tables II and III).

TABLE II.—(Group I.)

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
		Rabbit No. 6.			
4/9/20	$\frac{1}{2}$ Roux bottle killed bacilli	1820 grm.	*Nil*		
8/9/20		1680 ,,			
11/9/20		1800 ,,	1 in 80		
15/9/20		1850 ,,			
18/9/20		1890 ,,	1 in 80		
22/9/20		1960 ,,			
23/9/20				500×10^6 intravenously	Survived.
		Rabbit No. 7.			
4/9/20	$\frac{1}{5}$ Roux bottle killed bacilli	1445 grm.	*Nil*		
8/9/20		1640 ,,			
11/9/20		1640 ,,	1 in 40		
15/9/20		1710 ,,			
18/9/20		1730 ,,	1 in 40		
22/9/20		1870 ,,			
23/9/20				500×10^6 intravenously	Survived.
		Rabbit No. 9.			
4/9/20	$\frac{1}{10}$ Roux bottle killed bacilli	1490 grm.	*Nil*		
8/9/20		1560 ,,			
11/9/20		1530 ,,	1 in 20		
15/9/20		1530 ,,			
18/9/20		1560 ,,	1 in 20		
22/9/20		1730 ,,			
23/9/20				500×10^6 intravenously	Paralysed. Died on 6th day.
		Rabbit No. 21 (*Control*).			
23/9/20		1900 grm.		1000×10^6 intravenously	Died on 3rd day.
		Rabbit No. 22 (*Control*).			
23/9/20		1840 grm.		500×10^6 intravenously	Paralysis of hind legs on 3rd day. Recovered.

Post-mortem Findings.

No. 9.—Small intestine showed some œdema but no hæmorrhages. Large intestine appeared normal. Some hæmorrhages in appendix. Lungs, spleen, liver, kidney showed no macroscopic change.

No. 21 (control).—Some small hæmorrhages in right lung. Spleen, liver, kidney and suprarenals appear normal. Small intestine œdematous and injected. Large intestine shows no macroscopic change. Rabbit had coccidiosis.

TABLE III.—(Group II.)

Rabbit No. 10.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
4/9/20	$\frac{1}{10}$ Roux bottle killed bacilli	1380 grm.	*Nil*		
8/9/20		1500 ,,			
11/9/20		1510 ,,	1 in 80		
15/9/20		1500 ,,			
18/9/20		1520 ,,	1 in 80		
22/9/20		1680 ,,			
26/9/20				1000×10^6 intravenously	Paralysis of hind legs. Died 3rd day.

Rabbit No. 11.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
4/9/20	$\frac{1}{5}$ Roux bottle killed bacilli + 8·0 c.c. ox bile	1370 grm.	*Nil*		
8/9/20		1340 ,,			
11/9/20		1330 ,,	1 in 20		
15/9/20		1420 ,,			
18/9/20		1410 ,,	1 in 20		
22/9/20		1460 ,,			
26/9/20				1000×10^6 intravenously	Survived.

Rabbit No. 12.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
4/9/20	$\frac{1}{5}$ Roux bottle killed bacilli + 8·0 c.c. ox bile	980 grm.	*Nil*		
8/9/20		860 ,,			
11/9/20		860 ,,	1 in 40		
15/9/20		850 ,,			
18/9/20		855 ,,	1 in 40		
22/9/20		930 ,,			
26/9/20				1000×10^6 intravenously	Survived.

TABLE III.—(Group II)—*continued.*

Rabbit No. 23 (*Control*).

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
26/9/20.		1780 grm.		1000 × 10^6 intra-venously.	Died 2nd day.

Post-mortem Findings.

No. 10.—Lungs, kidney and suprarenals showed no change. Liver markedly congested. Small intestine, injection and œdema of mucous lining. Rabbit infected with coccidiosis.

No. 23.—Lungs show some small hæmorrhages. Congestion of liver, kidney and spleen. Large intestine showed no change. Small intestine injected and œdematous. No hæmorrhages to be seen macroscopically. Rabbit infected with coccidiosis.

It would appear, then, from these experiments that a certain degree of immunity to *B. dysenteriæ* (Shiga) can be produced in rabbits by the oral administration of killed vaccines, but the experiment as a whole is not completely satisfactory, owing to the failure (in Group 1) of 500 × 10^6 to kill the control and to the complicating coccidiosis infections. The ingestion of the killed bacilli resulted in the appearance of agglutinins in the serum in every case, but the titre was never a high one. Furthermore, a perusal of these figures shows that the degree of immunity attained does not run parallel with the titre of agglutinins. It will be seen also from the body weights recorded in these two tables, that the ingestion of enormous quantities of killed dysentery bacilli is practically without effect on the general health of the animal. Before proceeding to record the second series of experiments, some observations on the effects of ingestion of living dysentery bacilli and of repeated doses of the killed organism are appended.

Effect of Ingestion of Living B. dysenteriæ (Shiga) on the Rabbits.

In order to ascertain whether or not rabbits could be infected by the introduction *per os* of living bacilli, half a 24-hour Roux bottle culture of living dysentery bacilli was given to a rabbit by the mouth in one dose. The body weight, which on the day of ingestion, 11/9/20, was 1560 grm., fell to 1400 grm. on the 19th of the same month, and by 11/10/20 had still further declined to 1260 grm. Otherwise the animal appeared perfectly well. The examination of its serum for the presence of antibody (agglutinins) gave negative results throughout. Even the administration of large quantities of living dysentery bacilli to rabbits by the mouth is without effect. Similar investigations carried out by Shiga (1898) and Flexner and Sweet (1906) gave negative results also.

Result of a Series of Oral Immunisation Doses.

To determine the degree of immunity produced by a series of doses *per os*, three rabbits were taken (Nos. 2, 3 and 4) and given three doses of $\frac{1}{5}$ of a Roux bottle culture at intervals of ten days. Unfortunately rabbit No 1 died four days after the second dose from pneumonia, associated with *B. pyocyaneus*, and rabbit No. 4 became infected with an organism of the hæmorrhagic septicæmia group, and had to be discarded. The remaining animal, No. 3 (1780 grm.) was inoculated intravenously with 2000×10^6 living *B. dysenteriæ* (Shiga) on the twenty-fifth day after the last ingestion of vaccine, and at the same time a control rabbit (1700 grm.) received the same dose. The control animal died the third day after the test dose, and rabbit No. 3 became paralysed on the third day and died on the fourth. The post-mortem findings in the control rabbit were much more severe than in the immunised one. Whereas No. 3 merely showed some œdema of the small intestine with one small hæmorrhage in the appendix, the control showed injection and a more marked œdema of the intestinal mucous membrane, with numerous hæmorrhages in the ascending colon, in which was also some diphtheritic ulceration. The lungs showed some small hæmorrhages, and the liver, kidneys, suprarenals and spleen were acutely congested, the latter organ being much enlarged (three times the normal size). *B. dysenteriæ* (Shiga) was isolated from the gall-bladder and spleen, but not from the contents of the large intestine. Although the post-mortem findings point to much more extensive damage in the control rabbit, it must be admitted that none of the other control animals employed in this investigation showed such marked changes. This experiment must be considered, therefore, as inconclusive.

SECOND SERIES OF EXPERIMENTS.

Comparison between the Immunity produced by Ingestion and by Subcutaneous Inoculation.

Since the discovery of *B. dysenteriæ* by Shiga, a practical method of prophylactic inoculation against dysentery has been the subject of many investigations. The high degree of toxicity of this organism has rendered the solution of this problem difficult, and many efforts have been made to reduce the toxicity of the vaccine without interfering with its power to confer immunity. Shiga conceived the idea of inoculating a mixture of killed bacilli and anti-serum. The introduction of sensitised vaccines on the principle of Besredka carried the idea a stage further, and experiments in this connection by Dopter (1909) and Broughton-Alcock (1914) may be here cited. The latter reported good results obtained with a vaccine, the toxicity of which had been reduced by treating the bacterial bodies with heated normal serum. Gibson (1917), Ruffer and Willmore (1918) amongst others have added to our knowledge in this field, and more recently the efficacy of lipo-vaccines has been investigated by Whitmore and Fennel (1918) and Olitsky (1918). In most of this work the titre of antibody has been accepted as a measure of the degree of immunity obtained, but evidence is accumulating that the two do not run a parallel course. From a comparative study of the degree of active immunity produced by different types of dysentery vaccines (heat killed, carbolised, treated with normal or with immune serum), J. D. Thomson (1916) concluded that the carbolised vaccine, whilst giving rise to the least reaction, was productive of the highest degree of immunity. It was decided, therefore, to compare the protection afforded by oral immunisation with that resulting from the subcutaneous inoculation of carbolised vaccines.

Preparation of carbolised vaccine.—This was prepared in the usual manner by suspending the 24-hour growth on agar of *B. dysenteriæ* (Shiga) in 0·5 per cent. carbolic acid in saline. The suspension was counted and the doses required made up to 1·0 c.c. with saline as required.

Administration of vaccine per os.—In these experiments the vaccine was delivered into the stomach by means of a small rubber cannula to which was affixed a small filter funnel. In this experiment 24 rabbits of suitable size were taken and divided into 2 series of 12 each. Series 1, which received the vaccine *per os*, was further subdivided into two groups, A and B, which were treated as follows:

SERIES I.

Group A : Rabbits Nos. 27, 28, 29, 30, 31 and 32.

Each rabbit in this group received three doses by the oral route of a suspension of *B. dysenteriæ* (Shiga), killed by heating to 60° C. The dose in each case was ⅕ of a Roux bottle culture and the interval between the dose ten days.

Group B : Rabbits Nos. 33, 34, 35, 36, 37 and 38.

These rabbits were immunised by the oral route, receiving similar doses to those given to Group A, with, however, the addition of 8·0 c.c. of sterile ox bile with each dose of vaccine. One of these animals—No. 35—died three days after the first dose from pneumonia.

SERIES II.

This series consisted of 12 rabbits which were immunised by subcutaneous inoculation of a carbolised vaccine of dysentery bacilli (Shiga). Three doses were given, one of 50 million and two of 100 million, the interval between the doses being ten days. Rabbits Nos. 40 and 52 died five days after the first inoculation, post-mortem examination revealing some congestion of the right lung, with œdema and injection of the small intestine.

The surviving animals of these two series were tested for their immunity to living *B. dysenteriæ* (Shiga) three weeks after the last immunising dose. The test doses employed were 1000 millions and 2000 millions, and were given intravenously. The results of this experiment are tabulated below:

TABLE IV.

No. of rabbit.	Immunisation.	Test dose.	Result.
27	3 doses (⅕ Roux bottle) per os	1000 million	Died on the 5th day.
28	,,	,,	Died on the 5th day.
29	,,	,,	Survived.
33	,, + ox bile	,,	Died on the 5th day.
34	,, ,,	,,	Died on the 5th day.
30	3 doses (⅕ Roux bottle) per os	2000 million	Survived.
31	,,	,,	Died on the 2nd day.
32	,,	,,	Died on the 5th day.

TABLE IV—*continued.*

No. of rabbit.	Immunisation.	Test dose.	Result.
36	3 doses ($\frac{1}{5}$ Roux bottle) + ox bile	2000 million	Died on the 2nd day.
37	,, ,,	,,	Survived.
38	,, ,,	,,	Paralysed—recovered.
39	Subcutaneous inoculation of carbolised vaccine, 3 doses (50, 100 and 100 million)	1000 million	Survived.
41	Ditto	,,	Survived.
42	,,	,,	Survived.
43	,,	,,	Survived.
44	,,	,,	Survived.
47	,,	2000 million	Survived.
48	,,	,,	Survived.
49	,,	,,	Survived.
50	,,	,,	Survived.
51	,,	,,	Died on the 5th day.
	Body weight.		
54	Controls 2000 grm.	500 million	Survived.
57	1450 ,,	,,	Died on the 3rd day.
55	1860 ,,	1000 million	Died on the 2nd day.
58	1974 ,,	,,	Died on the 2nd day.
56	1700 ,,	2000 million	Died on the 2nd day.
59	2000 ,,	,,	Died on the 3rd day.

The surviving rabbits were observed over a period of three weeks. During the first two weeks there was some loss of weight, but by the end of the third week an increase in weight was recorded.

Post-mortem findings.—In the case of the control rabbits the mucous membrane of the small intestine was œdematous and injected. Nos. 55, 58 and 59 showed some hæmorrhages in the lungs. Those rabbits immunised *per os* which succumbed to the test-dose showed the same changes as the control animals though they were less acute. Rabbit No. 51, the only animal of Series II which died after receiving the test dose, showed rather severe changes. Hæmorrhages were present both in the lungs and large intestine.

DISCUSSION.

From the above experiment it will be seen that while the rabbits immunised *per os* had acquired a certain degree of resistance to the intravenous inoculation of *B. dysenteriæ* (Shiga) (3 survivals out of 11 possibles), a much more solid immunity (9 survivals out of 10 possibles) had resulted from the subcutaneous inoculation of a carbolised vaccine. It is noteworthy also that the ingestion of bile did not seem to increase the efficacy of oral immunisation. Besredka drew attention to this point also, and states that the addition of bile was without result in the immunisation against *B. dysenteriæ.* It might be pointed out also, that in the first series the oral immunisation was carried out by feeding the rabbits with the killed bacilli, whereas in the second series the vaccine was introduced into the stomach by means of a cannula. In neither case did any solid immunity result.

CONCLUSIONS.

(1) It has been possible to produce a certain small degree of immunity in rabbits by the oral administration of *B. dysenteriæ* (Shiga).

(2) The immunity so obtained is found to be far inferior to that produced by the subcutaneous inoculation of a carbolised vaccine administered in three doses of 50, 100 and 100 millions.

(3) The oral administration of large quantities of killed dysentery bacilli to rabbits is without effect on their general condition as judged by the body weight.

To many members of the Bacteriological Staff of this Institute I am indebted for helpful advice and criticism.

REFERENCES.

Besredka, A.—(1919) "Du mécanisme de l'infection dysentérique de la vaccination contre la dysentérie par la voie buccale et de la nature de l'immunité antidysentérique," *Ann. de l'Inst. Past.*, **33**, 301.

Broughton-Alcock, W.—(1914) "Antidysenteric Vaccination," *Brit. Med. J.*, **1**, 306.

Dopter, C.—(1908) "Vaccination antidysentérique expérimentale par les voies digestives," *Compt. rend. Soc. biol.*, **64**, 868.—(1909) "Vaccination preventive contre la dysentérie bacillaire (ses bases expérimentales)," *Ann. de l'Inst. Past.*, **23**, 677.

Ehrlich, P.—(1908) "Historisches zur Frage der Immunisierung per os," *Wien. klin. Woch.*, 652

Flexner, S., and Sweet, J. E.—(1906) "The Pathogenesis of Experimental Colitis, and the Relation of Colitis in Animals and Man," *J. Exper. Med.*, **8**, 514.

Gibson, H. G.—(1917) "A New Method of Preparation of a Vaccine against Bacillary Dysentery which Abolishes Severe Local Reaction; also Experiments with this Vaccine on Animals and Man," *J. Roy. Army Med. Corps*, **28**, 615.

Hida, O., and Toyoda, H.—(1907) "Active Immunisierung per os," *Cent. f. Bakt.* Abt. I, Ref. **42**, 418.

Olitsky, P. K.—(1918) "An Experimental Study of Vaccination against Bacilli dysenteriæ," *J. Exper. Med.*, **28**, 69.

Ruffer, M. A., and Willmore, J. G.—(1908) "The Production of Immunity against Dysentery Toxin," *Brit. Med. J.*, **2**, 1176.

Shiga, K.—(1898) "Ueber den Dysenteriebacillus (*Bacillus dysenteriæ*)," *Cent. f. Bakt.*, Abt. I, Ref. **24**, 913.—(1908) "Ueber die aktive Immunisierung per os," *ibid.*, Abt. I, Ref., **42**, 41.

Thomson, J. D.—(1916) "Attempts to Produce a Safe and Effective *Bacillus dysenteriæ* (Shiga) Vaccine for Prophylactic Purposes," *Brit. Med. J.*, **1**, 303.

Whitmore, E. R., Fennel, E. A., and Petersen, W. F.—(1918) "An Experimental Investigation of Lipovaccines," *J. Amer. Med. Assoc.*, **1**, 427.

Zeitlin.—(1905) Communication to Discussion Reported in *Cent. f. Bakt.*, Abt. I, Ref., **36**, 24.

THE COMPARATIVE INFLUENCE OF PURE AND COMMERCIAL SUGARS AND OF COMBINED AND SEPARATE STERILISATION ON BACTERIAL METABOLISM.*

C. G. L. WOLF.

From the John Bonnett Memorial Laboratory, Addenbrooke's Hospital, Cambridge.

Received for publication October 24th, 1921.

FOR a long time bacteriologists who have been concerned with the differentiation of bacterial groups or subgroups by means of fermentation reactions have attempted to ensure the normality of their results by securing sugars from well-known makers of fine chemicals. In very few cases, indeed, have the sugars been re-purified or their purity tested by the workers examining them.

When one realises the technical difficulties of purification and of analysis this is quite understandable, and the samples of sugars, such as glucose and levulose, sent out by the best firms as chemically pure go to show that even the makers have not found the task of purification easy.

Most workers have recognised that the sugars with which they worked were not chemically pure even when so labelled. The colour and turbidity of the solutions were evidence of this. It can be said generally that the purity of carbohydrates as bought in the market is far behind the standard exacted for a similar class of inorganic chemicals.

The Carbohydrate Laboratory of the Department of Agriculture of the United States has recognised the disability of bacteriologists in this regard, and has sought to remove it by devoting some time to the methods of carbohydrate purification. This has been done with such success that it is now easy to prepare glucose (Hudson and Dale), fructose (Hudson), xylose (Hudson and Harding), galactose (Mason) and other sugars in a very high state of purity by the methods of Hudson and his collaborators.

Certain firms are also taking up the preparation of pure sugars and endeavouring to set standards of purity. Two recent papers, one by Pfanstiehl and Black (1921) and another by Graber (1921), give an indication of what can be done in this respect. With Schmit-Jensen (1920) they point out that none of the chemical tests are to be compared in delicacy with the biological tests. Conversely, therefore, sugars should be in a high state of purity if they are to be used for detecting fine differences in bacterial behaviour.

That small amounts of impurities may affect fermentation reactions is shown by the recent work of Schmit-Jensen, who has experimented with the rarer types of sugar by means of his ingenious micro-fermentation tubes. By the use of an organism such as *B. suipestifer* acting on xyloses he has been able to show that so small a concentration as 0·025 per cent. in bouillon will manifest itself by the production of gas.

---

* A report to the Medical Research Council.

With regard to the uncertainty existing in the differential fermentation of bacteria, it is difficult to make out whether the differences obtained by individual workers have been due to impurities in the sugar employed, to variations in the methods of preparing the media or to variations in the organisms themselves. We know that in the preparation of so complex a mixture as a bacteriological medium many factors enter which are difficult to control. One has only to recall the profound difference obtained by using different samples of peptone in the preparation of diphtheria toxin to realise that even now many of these factors are not resolved.

Certain workers have, however, paid some attention to the question of the purity of sugars employed for bacteriological purposes. Elser and Huntoon (1909), in their study of the meningococcus, go into the matter at some length. They used samples of sugar obtained from Kahlbaum and from Merck, without, however, attempting to purify them or to analyse them. They point out the ease with which galactose is altered by treatment with the smallest quantities of alkali, and that, in the case of dextrin, the various samples of this carbohydrate gave quite different results, due, as they believe, to the presence of impurities. It will be noted that dextrin has given anomalous results with the meningococcus in the hands of different observers.

Inulin is another carbohydrate of great utility when pure, but it is so often contaminated with fructose that a pure sample is difficult to obtain. The time of harvesting the artichokes is of great importance in securing the pure polysaccharide.

Differences in the behaviour of the gonococcus with galactose have been observed by various workers, and Elser and Huntoon showed that the fermentation observed by Dunn and Gordon did not take place if the sugar was sterilised separately from the bouillon, and the mixture effected aseptically after sterilisation of both.

With *B. diphtheriæ* similar differences have been encountered: thus Simmons (1920) found organisms with all other characteristics similar from human and feline cases of diphtheria which behaved differently to saccharose.

With regard to the influence of sterilisation on media containing glucose, there is equal reason to pre-suppose that one might find differences in the behaviour of media prepared in the ordinary routine way and those to which the sterile sugar solution has been added after sterilisation of the bouillon. I have observed in previous experiments that there is a loss in amino-acid content when sugar and tryptic broth are sterilised together, and have explained it as partly due to the Maillard (1913) type of condensation of amino acid and glucose. This loss is well seen in the present experiments. The change in hydrogen ion concentration was detected by the older bacteriologists in titrating media before and after sterilisation, and has been recorded many times since. That the effect of combined sterilisation may have important practical bearings is shown by the results of Elser and Huntoon on the gonococcus, and especially the meningococcus. With the latter organism no fermentation took place with either galactose or fructose when the sugar was sterilised separately in solution in distilled water. Lobry de Bruyn and Alberda van Ekenstein (1895) have shown that small quantities of alkali and heat will change a part of fructose to glucose; and, indeed, Kendall, Day, Walker and Ryan (1919) have recently asserted that it is useless to perform tests with fructose when the results with glucose are known, owing to the stereo-chemical lability of these sugars when the solvent is alkaline (see Wohl, 1893, 1897).

In view of the above it seemed worth while to ascertain whether, with some of the more common organisms, a quantitative difference could be made out when pure and commercial sugars were employed. As the sterilisation of sugars with the medium had been shown to yield definite qualitative differences with organisms such as the meningococcus, an investigation of this factor was undertaken at the same time.

In the course of work which had been done in my laboratory on the fermentation of organisms, it has been found that a very good degree of quantitative uniformity could be made out when the same organism was allowed to ferment under identical conditions. The quantitative method ought therefore to show up any difference due to the purity or treatment of the carbohydrate employed which would not be apparent with ordinary fermentation tests.

I was fortunate, through the kindness of Dr. C. F. Walton, Jr., of the Carbohydrate Laboratory in Washington, in being able to obtain from him a sample of chemically pure glucose prepared by the method of Hudson. This was used in part of the work. In addition I recrystallised a good sample of "chemically pure" glucose from glacial acetic acid by this method. The recrystallisation was repeated. The product did not differ from that supplied me by Dr. Walton. It was snow-white, and gave an absolutely water-white solution when dissolved in water. It had been heated to 80° C., and subsequently kept *in vacuo* over sulphuric acid and potassium hydroxide for a long time.

The lactose was prepared from the sample which was used as "commercial" lactose. It was recrystallised three times from boiling distilled water after decolorisation with animal charcoal. This sugar also gave an absolutely water-white solution.

The commercial samples of glucose and lactose were obtained from a well-known chemical supply firm, and came labelled "chemically pure." The glucose had a distinct yellow tinge. The solution of the sugar was light brown in colour. The lactose had much the same external qualities as the glucose. Both samples were of the quality usually employed in bacteriological laboratories.

In the preparation of the sugars for addition to the medium the following method was employed: A 20 per cent. solution of glucose was made up in distilled water, and made faintly acid to Congo red with hydrochloric acid. This was tubed off in lots of 20 c.c. and sterilised at 120° C. for 30 minutes.

The lactose was also dissolved in the same concentration in distilled water and sterilised without the addition of acid. Both solutions were colourless after treatment with heat. The commercial sugars were treated in the same way. They gave light to darkish brown solutions. The fermentations were carried out in long-necked 600 c.c. kjeldahl flasks (Harris, 1919). Where sugars were added after sterilisation of the medium, this was done aseptically by adding the contents of the sugar tube to the medium. The flasks were allowed to stand at 37° C. for 24 hours to test for sterility. After fermentation any evaporation was allowed for by diluting the fermented media to a definite volume. With the exception of *B. murisepticus* all fermentations were carried out anaërobically in order to follow easily the gas evolution.

The organisms employed were *B. coli*, *B. welchii*, *B. murisepticus* and *B. tetani*.*

These organisms were chosen because in *B. coli* and *B. welchii* we have bacteria which are not sensitive to the *milieu* in which they develop. *B. tetani* and *B. murisepticus* are usually assumed to be of somewhat fastidious growth habit.

The method of testing was to set up a series of flasks so that under identical conditions and with the same quantity of inoculum there were the following media:

(1) Basic medium with pure sugar added aseptically.
(2) Basic medium and pure sugar sterilised together.
(3) Basic medium with commercial sugar added aseptically.

The three flasks were kept at a constant temperature in one thermostat. In another at the same temperature duplicates of (1) and (2) were fermented.

The basic medium used was the tryptic broth of Cole and Onslow, which was diluted 1 : 3, giving a nitrogen content for the finished medium of about 0·3 per cent.

Table I (experiments A–E) gives the results of two sets of tests with pure glucose and *B. coli* where the sugar had been added before sterilisation (A and C) and after sterilisation (B and D) with a control when ordinary glucose had been used (E). For the inoculum a culture of *B. coli* was used which had been passed twice through peptone at 24-hour intervals and once at a 10-hour interval.

FERMENTATION OF *B. COLI* IN GLUCOSE PEPTONE.

The effect of sterilisation on the medium.—As a result of combined sterilisation one finds that the ammonia and volatile acids are slightly higher, but the most pronounced effect is that on the amino-acid concentration. There is a very marked disappearance of amino-acid groups as the result of heating the nitrogen containing molecules with the sugar. The extent to which this occurs depends apparently on the composition of the medium, the extent of heating, and other factors which have not been studied, for in the second glucose medium used for *B. welchii* the process has not gone on so far. This is probably due to the amount and character of the preliminary tryptic hydrolysis. A discussion of this would lead far, but the recent experiments of Onslow (1921) seem to show that tryptic digestion is not easily controlled, for some workers obtain yields of free tryptophane on simple tryptic digestion which in other circumstances are only given by a second digestion. Onslow's experience has been confirmed in this laboratory.

---

* For *B. murisepticus* and *B. tetani* I am indebted to Dr. J. C. G. Ledingham, Director of the National Collection of Type Cultures, Lister Institute. *B. coli* was a human type isolated by Dr. Wyeth in my laboratory and used by him in his studies of this organism. *B. welchii* was one of the strains used by me in former work.

The volatile acids are higher, as are the fixed acids. To this may be ascribed the increase in hydrogen ion concentration. In this experiment the volatile acids have roughly been doubled as the result of sterilisation.

The condensation of glucose is shown by a loss of 0·07 per cent. on an initial concentration of 1·87 per cent.

The effect of sterilisation on fermentation.—While with the added sugar, both pure and commercial, there is a marked consumption of amino nitrogen, amounting in the former case to 40 mgrm., the amount of this component is larger at the end of fermentation when sugar and medium have been sterilised together. It would appear that the organism has attacked the condensation products of sugar and amino acids, and liberated part of the latter. The two experiments are quite concordant. Volatile acid formation is always higher where sugar has been added separately.

The composition of the evolved gases varies within the fairly narrow limits of 49·8 and 53·8 per cent. for carbon dioxide. It does not appear that sterilisation of the sugar with the tryptic broth has influenced this phase of the reaction. The total evolution of gas also does not seem to have been influenced either by sterilisation or by the quality of the sugar. The total variation is 43 c.c., the highest amount being evolved by the impure glucose.

No real difference in final reaction is to be made out in the various experiments, the resulting pH being 4·6. The consumption of sugar is slightly less in the experiment where glucose and medium have been sterilised together.

FERMENTATION OF B. COLI WITH LACTOSE PEPTONE.

The influence of sterilisation on the medium.—As shown in Table II, the effect on the lactose medium, as might be expected, is not so pronounced as with glucose, but, in general, heating has produced changes in the same direction. The ammonia is slightly lower in the combined sterilisation, but the change is not well marked in any. There is a small disappearance of amino acid in this medium, but this is confused by the result obtained with impure glucose where the amount of amino nitrogen is lowest. It will be noted that the results obtained in the second experiment with *B. welchii* in this medium are slightly in the other direction. It is therefore safe to assume that any effect on the amino nitrogen is negligible.

The volatile acids are undoubtedly higher, agreeing well with the control media in the *B. welchii* experiment. The fixed acids are practically unchanged by the heating. There has been a destruction of lactose as the result of heating, amounting to about 0·2 per cent. There is not the same increase in hydrogen ion concentration observed with the glucose medium, the increase amounting to pH = 0·3. In the *B. welchii* experiment the difference was pH = 0·4.

The effect of sterilisation on fermentation.—The amount of gas evolved does not seem to have been influenced by sterilisation or by the impurity of the

TABLE I.—B. coli *in* 2 *per cent. Glucose-peptone.*

	Pure glucose and peptone sterilised together.			Pure glucose and peptone sterilised separately.			Commercial glucose and peptone separately.	
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.	Initial.	Final.
Total nitrogen per cent.	0·325	0·321	0·323	0·327	0·323	0·332	0·324	0·336
Ammonia nitrogen: mgrm. per 100 c.c.	19·05	29·0	30·0	15·9	32·1	30·8	18·2	31·8
Amino acid nitrogen: mgrm. per 100 c.c.	80·0	96·4	96·2	120·5	80·0	80·6	118·0	90·7
Volatile acids: c.c. N/10 acid per 100 c.c.	7·4	20·7	21·6	3·8	25·2	22·2	3·61	23·5
Fixed acids: c.c. N/10 acid per 100 c.c.			26·3					
Carbon dioxide per cent.		50·9	50·8		53·8	50·6		49·8
Hydrogen per cent.		49·1	49·2		46·2	49·4		50·2
Total gas: c.c. in 47 hours		241	232		221	253		264
pH 20° C.	6·60	4·64	4·64	7·15	4·70	4·65	7·20	4·69
Glucose	1·89	1·22	1·22	1·87	1·25	1·20	1·86	1·19

TABLE II.—B. coli *in* 2 *per cent. Lactose-peptone.*

	Pure lactose and peptone sterilised together.			Pure lactose and peptone separately.			Commercial lactose and peptone separately.	
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.	Initial.	Final.
Total nitrogen per cent.	0·336	0·326	0·326	0·320	0·333	0·326	0·314	0·342
Ammonia nitrogen: mgrm. per 100 c.c.	17·9	30·2	30·9	19·0	21·2	31·1	18·6	26·7
Amino acid nitrogen: mgrm. per 100 c.c.	120	109	105	125	116	119	111	118
Volatile acids: c.c. N/10 acid per 100 c.c.	5·5	26·7	23·4	3·4	25·5	24·2	3·2	25·7
Fixed acids: c.c. N/10 acid per 100 c.c.	13·1	41·7	42·0	14·5	41·7	46·7	9·0	41·2
Carbon dioxide per cent.		46·1	46·1		52·1	45·7		52·5
Hydrogen per cent.		53·9	53·9		47·9	54·3		47·5
Total gas: c.c. in 48 hours		286	282		304	294		280
pH 20° C.	6·92	5·01	4·96	7·22	5·01	4·93	7·25	5·00
Lactose	1·54	1·11	1·11	1·72	1·13	1·15	1·69	1·21

glucose. It is of the same order as that produced from glucose. The final concentration of sugar is nearly the same in all experiments with the exception of the commercial glucose, where it is slightly higher. Owing to the initial concentration of lactose in the combined sterilised medium being low, the consumption of sugar in this medium is less than in the one where sugar was added before sterilisation.

FERMENTATION OF B. WELCHII WITH GLUCOSE PEPTONE.

The inoculum was prepared by transferring *B. welchii* through two meat tubes to milk for 24 hours, and from this medium to glucose peptone for 14½ hours; 0·7 c.c. was used for inoculation. The growth was somewhat slimy in character.

The effect of fermentation on the production of ammonia in the pure glucose media is entirely negligible (Table III), while there is an increase in the amount of amino nitrogen, due to the action of the bacteria. The amino-acid formation is the same in amount in both combined and separate media. The result with ordinary glucose is not decisive, both initial and final values being the same. The amount of volatile acids is not sufficiently affected to show any difference due to the method of sterilisation, and the same may be said of the fixed acids. In one respect, however, combined sterilisation has affected the fermentation, and that is in the gas production. Here it is considerably lower, then, when glucose is added separately to the medium. *B. welchii* is considerably more sensitive in this respect than *B. coli*.

The final amounts of sugar remaining over after fermentation are the same. With the commercial sugar experiment, as the initial concentration in the combined sterilisation is lower, less sugar was consumed in these experiments.

The final hydrogen ion concentrations are all alike.

FERMENTATION OF B. WELCHII IN LACTOSE PEPTONE.

The results (Table IV) in this experiment do not call for any special comment. There are no striking differences to be made out. Both nitrogen and carbon metabolism do not appear to have been affected by changes in the medium due either to the purity of the carbohydrate or to the method of sterilisation. There appears to have been less sugar consumption and a lower hydrogen ion concentration in the medium where the sugar was sterilised separately from the bouillon.

FERMENTATION OF B. MURISEPTICUS IN GLUCOSE PEPTONE.

In this experiment (Table V), which was allowed to continue for fifteen days, only the effect of purity of the sugar was examined. Here a certain difference was encountered. The ammonia production was considerably higher when commercial glucose was employed, while the volatile acid production was slightly lower. I was unable to determine the fixed acid production. The resulting final hydrogen ion concentration of the experiments was absolutely in agreement.

TABLE III.—B. welchii *in 2 per cent. Glucose-peptone.*

	Pure glucose and peptone sterilised together.			Pure glucose and peptone sterilised separately.			Commercial glucose and peptone sterilised separately.	
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.	Initial.	Final.
Total nitrogen per cent.	0·325	0·325	0·321	0·319	0·332	0·334	0·320	0·328
Ammonia nitrogen: mgrm. per 100 c.c.	23·2	24·2	21·7	27·1	26·7	30·4	19·0	29·2
Amino acid nitrogen: mgrm. per 100 c.c.	99·4	110·5	106·6	109·1	124·6	121·0	121·5	122·0
Volatile acids: c.c. N/10 acid per 100 c.c.	5·54	34·1	29·8	2·26	30·3	31·0	2·8	?
Fixed acids: c.c. N/10 acid per 100 c.c.	9·8	28·2	41·5	8·4	34·9	37·5	9·0	37·5
Carbon dioxide per cent.		45·4	44·4		45·2	45·4		47·2
Hydrogen per cent.		54·6	55·6		54·8	54·6		52·8
Total gas: c.c. in 24 hours		267	300		410	400		415
pH 20° C.	6·17	4·43	4·46	6·90	4·43	4·45	6·95	4·48
Glucose	1·62	0·98	0·99	1·70	0·98	1·00	1·80	0·69

TABLE IV.—B. welchii *in 2 per cent. Lactose-peptone.*

	Pure lactose and peptone sterilised together.			Pure lactose and peptone sterilised separately.			Commercial lactose and peptone sterilised separately.	
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.	Initial.	Final.
Total nitrogen per cent.	0·206	0·208	0·210	0·201	0·208	0·212	0·203	0·216
Ammonia nitrogen: mgrm. per 100 c.c.	15·3	8·9	19·5	14·8	31·3	24·3	15·4	36·6
Amino acid nitrogen: mgrm. per 100 c.c.	98·0	111·0	109·0	93·8	98·6	103·0	98·5	103.0
Volatile acids: c.c. N/10 acid per 100 c.c.	4·0	29·6	28·6	2·5	30·8	30·2	2·5	36·2
Fixed acids: c.c. N/10 acid per 100 c.c.	4·6	30·8	30·7	3·8	28·8	32·8	7·65	34·5
Carbon dioxide per cent.		43·7	47·2		44·9	46·5		47·2
Hydrogen per cent.		56·3	52·8		55·1	53·5		52·8
Total gas: c.c. in 48 hours		405			425	421		420
pH 20° C.	6·80	4·72	4·69	7·22	4·98	5·00	7·15	5·01
Lactose	1·60	1·09	1·11	1·62	1·26	1·24	1·59	1·25

Table V.—B. murisepticus *in* 1 *per cent. Glucose-peptone.*

	Pure glucose and peptone.		Commercial glucose and peptone.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·220	0·220	0·220	0·220
Ammonia nitrogen: mgrm. per 100 c.c.	13·6	53·3	13·6	78·5
Amino acid nitrogen: mgrm. per 100 c.c.	100·0	108·5	100·0	107·7
Volatile acids: c.c. N/10 acid per 100 c.c.		18·2		15·5
pH		4·60		4·60

FERMENTATION OF B. TETANI IN GLUCOSE PEPTONE.

Two experiments were made with *B. tetani*, one lasting 143 hours and the other lasting 323 hours (Table VI). The organism was sown on cooked meat and an inoculum of 0·6 c.c. used.

Table VI.—B. tetani *in* 1 *per cent. Glucose-peptone.*

	Pure glucose and peptone.			Commercial glucose and peptone.		
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.
Total nitrogen per cent.	0·242	0·238	0·223	0·247	0·239	0·212
Ammonia nitrogen: mgrm. per 100 c.c.	15·1	65·1	59·3	15·4	65·0	64·0
Amino acid nitrogen: mgrm. per 100 c.c.	110·0	49·3	71·1	113·0	49·4	74·2
Volatile acids: c.c. N/10 acid per 100 c.c.		32·0	28·5		31·7	31·0
Total gas evolved: c.c.		44·4	64·2		39·7	63·2
Carbon dioxide per cent.			61·3			64·1
Hydrogen per cent.			38·7			35·9
pH						6·9

The fermentation, as shown by gas evolution, was very slow, only about 3 to 5 c.c. of gas being produced in the first twenty-four hours. From thereon the course of gas production rose until about the fiftieth hour, when evolution slowed up. The gas production in the second experiment was always somewhat in advance of the first. They were not conducted with the same inoculum. The noteworthy feature of the fermentation of *B. tetani* is the high ammonia formation and amino-acid consumption. This is typical of strongly proteolytic organisms, such as *B. sporogenes* and *B. histolyticus* (Wolf and Harris, 1918). There is no difference to be observed between the fermentations conducted with pure and with commercial sugars.

SUMMARY.

Four organisms, *B. coli*, *B. welchii*, *B. murisepticus* and *B. tetani*, have been examined to ascertain whether the purity of a sugar or the combined sterilisation of the sugar with the bouillon had an effect on the products of bacterial metabolism. The sugars used were glucose and lactose. While minor differences were encountered, these were not sufficient to indicate that combined sterilisation or commercial glucose or lactose would influence a fermentation reaction sufficiently to lead to error. At the same time it must be remarked that with organisms more sensitive than those employed in the present study it is quite possible that such errors could take place.

REFERENCES.

de Bruyn, L., and van Ekenstein, A.—(1895) *Rec. trav. Chim.*, **14**, 156.
Elser, W. J., and Huntoon, F. M.—(1909) *J. Med. Res.*, **20**, 373.
Graber, H. T.—(1921) *J. Indust. and Eng. Chem.*, **13**, 687.
Harris, J. E. G.—(1919) *J. Pathol. and Bacteriol.*, **23**, 30.
Hudson, C. S.—(1917) *J. Indust. and Eng. Chem.*, **10**, 176.
Hudson, C. S., and Dale, J. K.—(1917) *J. Amer. Chem. Soc.*, **39**, 320.
Hudson, C. S., and Harding, T. S.—(1917) *J. Amer. Chem. Soc.*, **39**, 1038.
Kendall, A. I., Day, A. A., Walker, A. W., and Ryan, M.—(1919) *J. Infect. Dis.*, **25**, 189.
Maillard, L. C.—(1913) 'Génese des matières protéiques,' Paris (Masson et Cie).
Mason, E. H.—(1921) *J. Biol. Chem.*, **47**, 176.
Onslow, H.—(1921) *Biochem. J.*, **15**, 392.
Pfanstiehl, C., and Black, R. S.—(1921) *J. Indust. and Eng. Chem.*, **13**, 687.
Schmit-Jensen, H. O.—(1920) *Kgl. vet og. Landbohojskoles Aarskrift*, 103.
Simmons, J. S.—(1920) *Amer. J. Med. Sci.*, **160**, 589.
Wohl, A.—(1893) *Ber. Deut. Chem. Ges.*, **26**, 730.—(1897) *ibid.*, **30**, 3101.
Wolf, C. G. L., and Harris, J. E. G.—(1918) *J. Pathol. and Bacteriol.*, **22**, 1.

SEPTICÆMIA: THE SELECTIVE DEPOSITION OF THE COLON TYPHOID GROUP OF BACTERIA IN FIXATION ABSCESSES.

T. H. C. BENIANS,

Pathologist to the Prince of Wales's General Hospital, London.

Received for publication November 8th, 1921.

THE constancy of the septicæmic stage early in an attack of typhoid fever, before extensive localising lesions are to be found, makes it probable that this organism can enter and presumably leave the blood-stream without the help of such gross mechanical lesions as would occur where thrombosis of vessels and suppurative disintegration of tissues has taken place. The great frequency of *B. coli* infections of the urinary tract, many of which are most probably hæmatogenous in origin, and which often set in without any marked general symptoms, further goes to show that the coliform bacilus also can enter and leave the blood-stream with comparative ease.

In the experiments detailed below it is shown that the coli-typhoid group of bacteria can in fact pass out of the blood-stream into certain materials introduced into the subcutaneous tissues, and continue their growth there, while other bacteria present in the blood-stream fail to do so.

Inasmuch as this group of organisms, whether in health or disease, is to be found growing in the mucus-secreting intestinal canal the fixation areas were first made with a mucoid substance—gum tragacanth—and were designed to find whether the coli-typhoid bacteria as distinguished from other organisms were specifically thrown out from the blood-stream into this substance. With few exceptions this seemed to be the case.

Preparation of the gum.—An emulsion of about 5 per cent. gum tragacanth in water will just pass through a fair-sized exploration needle, and this strength was used in all the experiments. The emulsion was made up in the usual way by moistening the powder with alcohol, and then rapidly mixing with the appropriate amount of water and shaking well. The emulsion is spoiled by autoclaving; and, in fact, to be ideal, should not be heated at all, but, if necessary, it can be sterilised by steaming for a few minutes on three consecutive days. Almost all the samples I have worked with have been infected with sporing bacilli.

Experiment 1.—The Fate of the Sterile Gum in the Subcutaneous Tissues.

When first injected the gum forms under the skin a rounded elastic lump, which in the course of a few days swells up a little and becomes more diffuse, then diminishes in size and becomes firmer, but does not entirely disappear. It ultimately becomes replaced with a mass of fibrous tissue, which may contain a small area of degenerate pus. As shown by puncture and examination of films, invasion by phagocytes begins within an hour or so after the injection, and becomes more intense as time goes on, although as a rule the mucoid character is retained for several weeks. If the animal is killed the day following injection leashes of dilated vessels are seen all over and about the gum, associated with a good deal of œdema.

Experiment 2.—Injection of Gum Infected with Living Bacteria.

The results to be obtained in this experiment vary considerably according to what organism is being used. Infection with living *B. coli* shows that this bacillus grows freely in the mass despite the leucocytic infiltration. The abscess grows larger in the first few days and softens, although it rarely breaks down sufficiently to involve the skin. After the first few days of active infiltration the abscess, as a rule, shrinks up during the succeeding two or three weeks, and then becomes partly or wholly organised. The definitely mucoid character of the contents is usually still marked up to the end of the first week.

Infection of the gum with living *S. aureus* gives a more active picture. The infiltration with phagocytes from the first is much more fierce; there is extensive œdema in the vicinity, and masses of lymph are deposited throughout the abscess, which is liable in a few days to break through the skin, discharge freely, and heal up completely. The staphylococcus, like the coliform bacillus, appears to multiply in the gum in the first day or two after injection. In both staphylococcal and coliform abscesses phagocytosis may be noted to a variable extent during the second or third day, but it is not usually a notable feature. It is noteworthy that while the coliform bacilli seem to remain unchanged as regards their staining characters, the staphylococci within a day or two often lose their capacity to retain Gram's stain, or stain with only a small central dot.

In addition to these two principal types of organisms, the various other bacteria referred to in the later experiments were also inoculated directly into gum masses and injected, and with the exception of the gonococcus they were recovered living from the abscesses on the following day. In most cases they appeared to have increased in numbers.

Experiment 3.—The Migration of B. coli *from the Blood-stream into a Subcutaneous Mass of Gum.*

A rabbit was injected subcutaneously in the flank with 5 c.c. of the sterile gum. At the same time about 2500 million living *B. coli* suspended in 1 c.c. saline were injected into the ear vein. Three hours after injection the gum mass was punctured with a sterile needle on a syringe and some of the contents withdrawn. The mucoid matter was slightly turbid, and films showed a few leucocytes and occasional Gram-negative bacilli. Several colonies of *B. coli* grew from a loopful of it in pure culture. Puncture after 6 hours showed the same conditions in a more marked degree. Puncture 24 hours after injection showed pus-cells and bacilli in very large numbers, and *B. coli* grew in a massive and pure culture. Occasional punctures showed the presence of living *B. coli* in the abscess up to the end of the second week, by which time the mucoid character had entirely disappeared, and the abscess had a dense fibrous wall with a purulent centre.

Living *B. coli* were obtained from the blood-stream up to the third day after injection.

The experiment shows that *B. coli* may leave the blood-stream and start within three hours to set up a local infection in a mass of sterile gum lying in the subcutaneous tissues. It further shows that *B. coli* may remain alive in the blood-stream for 72 hours, and in a localised abscess of the nature described for at least 2 weeks. (In a previous paper I (1920) have shown that Shiga's dysentery bacillus may be recovered alive from a similar abscess in the guinea-pig after 2 months.)

Organisms contained in a localised abscess of this type are obviously protected from the destructive agencies of the host, and this in spite of the presence of numerous phagocytes.

Experiment 4.—Repetition of Experiment 3, but using S. aureus *in place of* B. coli.

5 c.c. gum were injected subcutaneously in the flank, and 5000 million *S. aureus* were injected simultaneously into the blood-stream. The gum was punctured the next day and again the following day, and examined by film and culture. Large numbers of pus-cells were seen but no cocci, and both times cultures remained sterile. A similar puncture on the fifth day was also sterile. The experiment was repeated on another rabbit, with identical results.

We learn from this experiment that the *S. aureus* does not readily pass out of the vascular system to infect a mass of gum injected into the subcutaneous tissues, and in this respect it is in marked contrast to the coliform bacillus which was found in the gum within three hours after the injection. It seems that some special mechanism is in operation enabling the former to pass through the vessel walls.

Experiment 5.—Repetition of Experiments 3 and 4, using both Types of Bacteria together.

A rabbit was injected with gum as before, and a saline emulsion containing living cultures of *B. coli* and *S. aureus ;* 2500 million of each were at the same time given intravenously. Puncture of the gum after 3, 6 and 24 hours showed only bacilli in the abscess, and with the exception of one colony of *S. aureus*, obtained at the second puncture, pure growths of *B. coli* were obtained each time.

As it has been shown in Experiment 2 that either of these types of bacteria is able to grow in the gum when injected into it direct, it follows from Experiments 3, 4 and 5 that a definite selective capacity exists, which enables bacteria of the colon type to leave the blood-stream and infect the gum much more readily than those of the staphylococcal type.

Experiment 6.—Repetition of the previous Experiments, using various other Bacteria.

(*a*) Experiment 5 was repeated, using *B. paratyphosus B* instead of *B. coli*. This organism was recovered from the gum as in the case of *B. coli*, but, as before, the cocci were not found in it.

(*b*) A mixed emulsion of *B. typhosus*, *S. aureus* and Hofmann's bacillus was tested by intravenous inoculation in the same way, and only the typhoid bacillus was obtained from the gum.

(*c*) Other organisms similarly tested as regards transference from the blood-stream to the gum were *M. catarrhalis* and streptococcus, a mixed culture freshly isolated from the sputum. Neither of these organisms was grown in culture from the gum, although a few streptococci were seen in films made 24 and 48 hours after inoculation. Both were grown in blood cultures made 24 hours after injection into the blood-stream.

(*d*) A mixed emulsion of the influenza bacillus and streptococcus, freshly isolated from sputum, was tried, but neither organism was recovered from the gum. On the day following the injection only the streptococcus was grown in blood-culture (3 c.c. of blood).

These experiments, then, show that while the coli-typhoid organisms almost always appear in the fixation gum abscess, the various other bacteria, examined in the same way, almost invariably fail to be transferred from the blood-stream to the fixation area.

The recovery of the coli-typhoid bacilli did not, however, occur on every occasion without exception: thus out of 9 times that Experiment 5 was done *B. coli* was recovered in 5 of the experiments in pure culture from several punctures. In 2 other experiments staphylococci in small numbers were grown from the gum, in addition to *B. coli*. Once the gum remained sterile, and once *S. aureus* only grew from it.

Of 3 experiments with *B. typhosus* in company with *S. aureus*, once the cultures remained sterile and twice typhoid bacilli grew in pure culture from repeated punctures. The two experiments with *B. paratyphosus B* along with *S. aureus* both resulted in pure cultures of paratyphoid bacilli in the fixation abscesses.

Experiment 7.—Substances other than Gum used for the Fixation Areas.

Mucin.—Commercial mucin (scales) dissolved in dilute caustic soda and neutralised with HCl was used as a subcutaneous inoculum. As in the gum experiments, *B. coli* and *B. typhosus* were recovered from the mucin, whilst the staphylococci failed to appear in it. Only two experiments were carried out, as the technical difficulties in preparing the mucin sterile were considerable.

Starch.—A 5 per cent. emulsion of boiled starch was used in 3 experiments as a fixation area, with an intravenous inoculum of mixed *B. coli* and *S. aureus*. After 24 hours the starch showed a considerable number of phagocytes, but no organisms, and cultures remained sterile. The result after 48 hours was the same. In the third repetition of the experiment a few colonies of *B. coli* were grown from a puncture 48 hours after inoculation.

The presence of numerous phagocytes in the starch without any infecting organisms is an interesting comment on a possible suggestion that bacteria might be carried into the gum by these migrating cells.

Agar.—Two experiments were carried out using sterile 2 per cent. agar as the subcutaneous inoculum, injecting it when cold and solid. *B. coli* and staphylococci were also injected intravenously. After 24 hours a fair number of leucocytes had migrated into the agar, but no bacteria were seen and cultures remained sterile. In a second experiment a great number of bacilli were seen in films after 24 hours, and cultures grew an abundance of *B. coli* in pure culture.

McConkey's bile-salt agar.—The two agar experiments were repeated with this medium as a subcutaneous inoculum. Leucocytes migrated into it freely, and in both experiments an abundance of *B. coli* grew from the 24 hours' puncture. In the first experiment the coli were in pure culture, but in the second a few colonies of *S. aureus* also grew.

As control a further rabbit was injected with masses of sterile agar and McConkey's agar; these masses remained sterile but were freely invaded by phagocytes.

Muscle.—The pale hind limb muscles of a freshly-killed rabbit were finely smashed up in a mortar and injected subcutaneously into a rabbit, a mixed suspension of *B. coli* and *S. aureus* being given intravenously at the same time. The rabbit was found moribund 24 hours later and was killed. There was much inflammation in the region of the inoculum with an extensive œdema, which gave the whole district a watery mucoid appearance. The mashed muscle seemed very little changed and films showed only a few leucocytes invading it; large numbers of Gram-negative bacilli were present, but no cocci. *B. coli* grew abundantly in pure culture from the watery exudate. The experiment was not successfully repeated on account of the great difficulty in pushing the mashed muscle through a syringe and needle. This experiment again shows that *B. coli* leaves the blood-vessels more readily than does the staphylococcus, and as in the case of the starch experiment, but in the converse way, we see that the migration of the bacteria bears no relation to that of the phagocytes.

Egg albumen.—When injected in the raw uncoagulated state this substance was rapidly absorbed; a few phagocytes were withdrawn from the inoculation area by puncture, but none of the bacteria from the intravenous injection were recovered. Albumen coagulated by heat before injection seemed to be practically inert as regards invasion either by phagocytes, or bacteria from the blood-stream.

Fat.—Mutton fat used as a subcutaneous inoculum remained solid in the tissues, and, like the coagulated albumen, remained inert and free both from phagocytes and bacteria.

In a good many of the experiments in which staphylococci were injected intravenously the rabbits became definitely ill before the end of the first week; these when killed almost always showed numerous cortical abscesses in both kidneys. Films and cultures from these abscesses showed the presence of large numbere of staphylococci. In some cases coliform bacilli were also present in small numbers, but failed to grow in culture. I have no note of an abscess of this type being found in an animal infected only with coli-typhoid bacilli.

DISCUSSION.

How do the organisms find their way into the subcutaneous inoculum? From the fact that the migration in these experiments was limited almost entirely to the coli-typhoid bacteria, it seems that there must be some special characteristic or mechanism which facilitates their passage through the walls of the vascular system, presumably through the capillaries.

So far as we know there is no filtrable form of coliform bacilli which might readily pass out of the capillary walls, and in point of size they are mostly larger than the cocci. The actual motility of the organism might play a part in the case of some of the members of the coli-typhoid group, but the specimens of *B. coli* used in the experiments were not motile, so this mechanism must be ruled out. Mere mechanical lesions of the vessels is negatived in the first place by the fact that in mixed inoculations only one type of organism escapes. Moreover, in some of the experiments the intravenous injection was delayed for 24 hours after the introduction of the gum, so that closure of any ruptured vessels might have time to take place. Another possible way that organisms might migrate is through the medium of phagocytosis. We have seen that phagocytes invade both sterile gum and sterile agar, and the question arises whether they carry the bacilli out with them. We know that the coliform bacillus when inside the phagocyte is in a relatively safe position, as it is protected there from the lysins of the blood-serum. This, then, would be an ideal explanation if it were the true one. As it is the evidence already brought forward in this paper is decidedly opposed to this mode of infection. In addition to the fact that phagocytes may be present (as in experiment 7, with starch, in great numbers) without any bacteria is the fact that where phagocytosis was noted in the fixation abscesses it was never found in the early, but only in the later stages—usually after 24 hours.

The evidence, then, is against phagocytic transportation of the bacteria in these experiments, and we are left to the assumption of a specific spontaneous mode of migation of which no explanation is offered here.

It is possible that all bacteria might resemble the coliform bacilli in readily leaving the blood-stream were it not that the cocci, for instance, are held back by some special mechanism. For instance, O. Thomsen (1920) has shown that the agglutination of organisms in the peritoneal cavity effectually prevents them from obtaining egress to the blood-stream. This, however, seems to throw no light on the experiments of this paper, for the cocci were not agglutinated by the rabbit's serum.

CONCLUSIONS.

(1) Organisms of the coli-typhoid group, when embedded in mucilage, in the subcutaneous tissues of the rabbit or guinea-pig are protected from the defence mechanisms of the host, and may survive for some weeks.

(2) These organisms, when directly inoculated with gum into the sub-

cutaneous tissues, do not produce the intense local reaction with liquefaction of tissues that follows similar local infections with *S. aureus*.

(3) These organisms are able to leave the vascular system without there being any gross mechanical lesion of the vessels. This facility of migration does not appear in the case of *S. aureus* and various other bacteria.

(4) In experimental coli-typhoid septicæmia a subcutaneous fixation abscess of sterile gum tragacanth quickly becomes infected with these bacteria. Staphylococci, on the other hand, rarely gain access to the gum from the blood-stream.

(5) It is suggested that the ready artificial infection of the mucoid bodies by the coli-typhoid bacteria bears a close similarity to the infection of the mucous membranes of the body from the blood-stream, as in the enteric fevers. It is not shown here whether the bodies of this physical character receive the bacilli owing to a selective mechanism, or whether they merely favour their growth on account of the protection they afford them.

(6) The transportation of the bacilli in these experiments apparently was not effected by phagocytes.

REFERENCES.

BENIANS, T. H. C.—(1920) " A Record of an Inagglutinable Form of Shiga's Dysentery Bacillus Experimentally Derived from an Agglutinable Culture," *J. Pathol. and Bacteriol.*, **23**, 171.

THOMSEN, O.—(1920) " Experimental Studies of the Mode of Action of Antimeningococcal serum," *Acta Med. Scandinavica*, **54**, 65.

A STUDY OF HÆMOGLOBINOPHILIC BACTERIA BY AGGLUTINATION AND AGGLUTININ ABSORPTION.

H. B. MAITLAND AND GORDON C. CAMERON.

From the Department of Pathology and Bacteriology in the University of Toronto.

Forwarded for publication August 5th, 1921.

B. influenzæ has been prominently associated with epidemic influenza, but it is well established that the organism may be present in a variety of other pathological conditions and in healthy carriers. The identity or variation of strains from different sources is a matter of considerable interest, since it throws an indirect light on the *rôle* of *B. influenzæ* in the epidemic disease, and is a further step in elucidating the group of hæmoglobinophilic organisms. Pfeiffer recognised that *B. influenzæ* was but one type of this group, and termed other similar organisms "pseudo-influenza bacilli." They, too, have been shown to be pathogenic and have been found in carriers.

For this investigation we have used thirty-eight strains of *B. influenzæ* isolated from hospital patients during a period when no cases of epidemic influenza were seen. They were all Gram-negative bacilli requiring blood-pigment for growth. On plain blood agar they grew in very minute colonies, and caused no hæmolysis after incubation for three days at 37° C. Profuse growth was obtained on chocolate agar (meat infusion agar, neutral to phenolphthalein, with 4 per cent. of glycerine, and the addition of 4 per cent. of citrated blood just before use, while the medium was at boiling-point), and this medium was used as a routine for isolation and subsequent transfer.

Morphologically the strains were not at all alike. The differences were definite, and although one might pick out certain forms as types, this would hardly be justified, since some strains would fall between, and make the variation a gradation rather than a clearly marked grouping. Some were composed of small coccoid bacilli fairly uniform in size and shape, amongst which a few longer forms could be seen. Others showed slender bacilli whose length was 1·5 to 4 times the width, and in some the organisms were mostly much longer, the length six to eight times the width, a few shorter forms

TABLE I.—*Results of Cross-Agglutination.*

Organisms.	Origin.	Limit titre of sera (homologous in heavy type).							
		Wilson.	Kuryk.	Finklestein.	Ford, blood.	A. M.	Gliskmaune.	9.	E.
Wilson . . .	Antrum in rhinitis	**1280**	0	0	80	640	40	0	40
Wilson II . . .	,, ,, ,,	**640**	0	0	40	0	0	0	0
Arnsden . . .	Pneumonia	1280	0	20	80	640	640	0	20
A. M. . . .	,,	1280	0	20	80	**1280**	1280	0	20
Gliskmaune . .	Subacute bronchitis	1280	0	20	80	640	**2560**	0	20
Leslie, lung . .	Pneumonia-autopsy	640	0	0	0	320	1280	0	0
Kopf	Chronic bronchitis	320	0	0	80	640	0	0	0
Moore . . .	H.S.C. acute ,,	640	0	0	80	40	0	0	0
Delamere . . .	Pneumonia	160	0	0	80	1280	2560	0	0
Ford, blood . .	H.S.C. infected knee	80	0	0	**2560**	20	0	0	0
,, knee . .	,, ,, ,,	160	0	0	2560	80	160	0	0
Kuryk . . .	Pneumonia	0	**1280**	80	0	20	0	0	0
E	—	320	2560	320	320	80	40	320	**2560**
9	—	0	40	0	0	20	80	**2560**	80
Finklestein . .	Pneumonia	0	20	**2560**	0	0	0	0	0
Lee	,,	320	0	0	20	320	160	0	0
Somers . . .	,,	40	0	0	0	160	640	0	0
Wellman . . .	,,	0	0	0	0	160	80	0	0
Simmons . . .	,,	0	0	0	20	40	160	0	0
Madill . . .	,,	0	0	0	40	160	320	0	0
McGreagor . .	,,	0	0	0	20	80	320	0	0
Million . . .	,,	160	0	0	0	160	0	0	0
Bellamy . . .	,,	0	0	0	160	80	0	0	0
Davis. . . .	H.S.C. bronchitis	160	160	0	0	80	640	0	0
Burrows . . .	,, ,,	160	0	0	40	40	0	0	0
Jones. . . .	Pneumonia	40	0	20	0	40	0	0	0
Stead. . . .	Acute bronchitis	160	0	0	0	80	0	0	0
Gilchrist . . .	Chronic ,,	0	0	80	20	0	0	0	40
Leslie . . .	Sputum of autopsy case	0	0	0	0	0	0	0	0
Hamilton, mouse .	Pneumonia	0	0	0	0	0	0	0	0
,, sputum .	,,	20	0	0	0	0	0	0	0
Sophia . . .	,,	20	0	0	0	0	0	0	0
Bradley . . .	,,	20	0	0	0	0	0	0	0
Bowen . . .	,,	20	0	40	0	0	0	0	0
Morgan . . .	,,	0	0	80	0	0	0	0	0
Eisen . . .	Bronchiectasis	0	0	80	0	0	0	0	0
Copeland . . .	H.S.C. meningitis	0	0	0	80	0	0	0	0
Burt	Pneumonia	0	0	20	0	0	20	0	40

For strains marked "H.S.C." we are indebted to Dr. I. H. Erb, who isolated them at the Hospital for Sick Children.
Strains 9 and E were sent to us from the laboratory of Prof. Zinsser, College of Physicians and Surgeons, New York.

being also present. The staining was usually solid, but bipolar forms were seen and irregularity in the centre of longer bacilli.

The origin of the strains is mentioned in Table I. Those from patients with lobar pneumonia, with the exception of two or three, were obtained by culturing the sputum. The exceptions came from the lung at autopsy or from the peritoneal cavity of a white mouse in the course of typing the pneumococcus. The latter was the dominant organism in these cases, but from cultures on chocolate agar it was common to find colonies of *B. influenzæ*, and occasionally in the mouse *B. influenzæ* grew as profusely as the pneumococcus.

TECHNIQUE.

Each strain was isolated by subculturing one colony. Suspensions for agglutinating were made by washing the surface growth from chocolate agar with physiological saline, shaking to break up the clumps, centrifuging out the coarser particles and diluting to a standard density. One-tenth per cent. of formalin was added and the suspensions stored in the ice-chest. The agglutinating sera were obtained from rabbits, and had a titre of 1/1280 or 2560. For absorption of agglutinins killed organisms were left overnight at 37° C. in contact with the serum diluted 1 in 10.

RESULTS.

Table I gives the results of cross-agglutinating thirty-eight strains with eight sera. Dilutions were made from 1 in 20 to 1 in 2560, using seven tubes and doubling the dilution in each. They were incubated in a water-bath at 56° C. for two hours, and the end-point taken as the last tube in which clumping could be seen with the naked eye.

By examination of the figures the strains may be resolved into several groups. The first nine have much in common. The two Ford strains are separate from the others. Kuryk, Finklestein, strains 9 and E, are all different and not related to the others. Strain E is susceptible to agglutination, but has a very narrow antigenic value. The next thirteen are acted upon to a slight extent by two or more sera, but the titres are so much below those for the homologous organisms that these strains fall undetermined outside the former groups. Similarly the last ten strains are an undetermined group, almost untouched by any of the sera.

There was also a difference in the agglutinating value of the sera. Four were quite specific, and did not agglutinate any other strain in a dilution comparable to that of the homologous organism; in fact most strains were not agglutinated at all by them. The other four acted on numerous strains in low dilutions. They have more in common with them than have the four highly specific sera, but although they agglutinated a large portion of the strains to a slight extent, only with the first nine were the titres high enough to suggest a grouping.

These nine strains were investigated by agglutinin absorption. Wilson serum was absorbed by each in turn. The results are given in Table II. In each case a control was made at the same time as the absorbed serum.

TABLE II.—*Results of Absorption Tests using Wilson Serum.*

	Wilson serum absorbed by:									
	Wilson.		Wilson II.		Arnsden.		A. M.		Gliskmaune.	
	Abs. serum.	Cont. serum.	Abs.	Cont.	Abs.	Cont.	Abs.	Cont.	Abs.	Cont.
Wilson	0	640	0	640	160	640	640	1280	160	640
Wilson II	0	640	0	320			640	640	160	640
Arnsden	0	640	40	640	0	640	320	1280	80	640
A. M.	0	640	0	640	40	640	160	640	80	640
Gliskmaune	0	640	0	640	0	640	80	1280	0	640
Leslie, lung	0	160	0	320	0	320	320	1280	0	160
Kopf	0	160	0	160	40	160	320	320	0	160
Moore	0	320	80	320	0	320	160	320	80	320
Delamere	0	160	0	160			160	320		

TABLE II *(cont.)*.

	Wilson serum absorbed by:							
	Leslie, lung.		Kopf.		Moore.		Delamere.	
	Abs. serum.	Cont. serum.	Abs.	Cont.	Abs.	Cont.	Abst.	Cont.
Wilson	160	640	160	640	640	640	320	1280
Wilson II	80	320	160	320	160	320	640	640
Arnsden	80	640	320	640	320	640	80	1280
A. M.	80	640	320	640	160	640	80	640
Gliskmaune	0	640	640	640	320	640	160	1280
Leslie, lung	0	320	160	320	320	320	40	320
Kopf	40	160	0	160	80	160	320	320
Moore	160	320	320	320	0	320	0	320
Delamere	40	160	80	160	0	160	0	320

From these results strains Wilson and Wilson II, isolated from the same person at different times, appear to be identical; the second strain absorbs practically all the agglutinin from the serum of the first, and they react in the same way toward the serum absorbed by other strains. Arnsden is different, and forms a second type. The A. M. organism did not fully absorb, although repeated attempts were made. However, by using the A. M. serum it was found to differ from Arnsden, and is therefore a third type. This is shown in Table III.

TABLE III.—*Results of Absorption Tests using A. M. Serum.*

	Serum A. M. absorbed by:			
	A. M.		Arnsden.	
	Abs.	Cont.	Abs.	Cont.
Arnsden	0	1280	0	1280
Gliskmaune	0	1280	320	1280
A. M.	0	1280	640	1280

That Gliskmaune differs from Arnsden and the previous three is demonstrated by Tables III and I. However, it is identical with Leslie lung, and these two make a fourth type. Similarly Kopf, Moore and Delamere may be isolated. Thus in the first nine strains there are seven serological races.

The two strains from Ford, one from the knee and the other from the blood, are the same, but stand by themselves. So also do Kuryk, E, 9, and Finklestein. Thus in the fifteen strains whose serological character we were able to determine with the sera at hand twelve races were found.

In two instances the identical strains came from the same person. Gliskmaune and Leslie, however, are similar strains from different sources. And it is of interest to note that the two strains obtained from Leslie—one from the lung, the other from the sputum—are not alike.

As to the remaining twenty-three strains, we can only say that with eight sera no grouping was evident, suggesting that, like the first fifteen, they, too, possess a serological individuality.

Our results are in accord with the reports of others who have made similar experiments.

Bell (1920) investigated thirty-seven strains from throats of normal students and influenza cases by agglutination and agglutinin absorption, and found that although identical strains did occur, the group is heterogeneous. He could not make any distinction between strains from normal throats during a non-epidemic period and those from influenza cases. The morphology varied from coccoid to fairly long bacilli, but did not afford a reliable means of grouping and did not correspond to differences in agglutination.

Valentine and Cooper (1919) made similar tests on 171 strains with twenty-five sera, and found no serological relationship.

Povitsky and Denny (1921), working with 185 strains, obtained confirmatory results. Seven from meningitis cases had more in common than the others, four out of the seven belonging to one group.

The conclusions of Coca and Kelly (1921) supported the above findings.

Cohen (1909), comparing strains from meningitis and those from other sources, found that the former differed in agglutination and protection experiments, and produced a fatal septicæmia in rabbits.

CONCLUSIONS.

Viewing our results in the light of those from other laboratories, the following conclusions may be made:

(1) Nearly all strains of *B. influenzæ* possess a serological individuality as determined by agglutination and agglutinin absorption.

(2) Identical strains do occur, but they are not frequent.

(3) The antigenic value of strains varies. Some are extremely narrow. The serum prepared from such strains seldom agglutinated other organisms than the homologous.

(4) The morphological variations are marked, but do not correspond to variations in agglutination.

(5) Two or more serological races may be present in the same patient.

REFERENCES.

Bell, H.—(1920) "Relation of Different Strains of Influenza Bacilli as shown by Cross-Agglutination and Absorption Tests," *J. Infect. Dis.*, **27**, 464.

Coca, A. F., and Kelly, Margaret F.—(1921) "A Serological Study of the Bacillus of Pfeiffer," *J. Immunol.*, **6**, 87.

COHEN.—(1909) "La méningite cérébro-spinale septicémique," *Ann. de l'Inst. Pasteur*, **23**, 273.

POVITZKY, OLGA R., AND DENNY, HELEN T.—(1921) "Further Studies on the Grouping of Influenza Bacilli, with Special Reference to Permanence of Type in Carrier," *J. Immumol.*, **6**, 65.

VALENTINE, EUGENIA, AND COOPER, GEORGIA M.—(1919) "On the Existence of a Multiplicity of Races of *B. influenzæ*, as Determined by Agglutination and Agglutination Absorption," *ibid.*, **4**, 359.

THE RELATION BETWEEN BILE SALTS AND HÆMOLYSIS IN THE BLOOD-STREAM.

ERIC PONDER.

From the Department of Physiology, University of Edinburgh.

Received for publication September 14th, 1921.

THE fate of the bile salts in the blood in jaundice is an old and much-discussed question.

The normal amount of bile acids produced in man is about 10 grm. *per diem* (v. Noorden); yet only traces of bile salts are found in normal urine. In the first few days of an attack of jaundice there is evidence that an increased quantity of these substances is absorbed into the blood and excreted in the urine; but afterwards the amount excreted becomes diminished, since bile is excluded from the intestine, and therefore not absorbed. On the other hand, it is stated that in jaundice an increased amount of bile salts enters the blood, and thus produce increased biliary secretion, a vicious circle being established (Wells). In jaundice the presence of bile acids has been demonstrated in the blood (Croftan), and in the thoracic duct (Tapeiner).

It may therefore be taken that, in the first few days of jaundice at least, bile salts are absorbed into the blood-stream in increased amount. The question then arises: Why is there not hæmoglobinuria, and why is so little bile salt excreted in the urine?

Regarding the first part of the question, it is found that hæmoglobinuria is not a symptom in jaundice. A complete list of the conditions in which hæmoglobinuria occurs does not include this disease. It would be expected that, as bile salts are hæmolytic, their injection into the blood-stream would cause hæmoglobinuria; yet this does not appear to happen. That the bile salts could exert their hæmolytic action in the blood-stream seems to be accepted (Wells), and it has been supposed that they are present in insufficient quantity to cause any marked effect. In order to avoid the difficulty, the assumption has been made that part of the bile salts are destroyed in the blood; it has been conclusively shown, however, that all the bile acids absorbed from the intestine are re-secreted in the bile (Stadelmann). In about one-fourth to three-fourths of the cases of jaundice, ranging with the type, no bile salts are found in excess in the urine.

Observations recently published (Ponder) show that, contrary to the usual opinion, bile salts do not exercise their hæmolytic action in the presence of blood serum. The inhibitory action of serum is very great. The presence of 0·1 c.c. of serum lengthens the time required for the hæmolysis of 1 c.c. of a 5 per cent. suspension of thrice washed human erythrocytes by a 1 in 1000 solution of sodium taurocholate from ½ minute to 7 minutes at 37°. If sodium glycocholate be used as the hæmolysing agent the time is lengthened, in the case of a 1 in 1000 solution acting at 37°, from 2 minutes to 12 minutes. Since these salts do not attain, even in intense jaundice, a concentration of 1 in 1000, or anything approaching it, and since the amount of blood serum present is proportionately much greater, it may be concluded that bile salts exert no hæmolytic action in the blood-stream. This explains the absence of hæmoglobinuria, and also is confirmed by statements that the blood in jaundice, as regards its red cell content, is normal.

It remains to be seen what occurs to the bile salts in the blood-stream. They are known to enter into an adsorption compound with proteins, but this only occurs in the presence of acid. It may be concluded that as the reaction of the blood is not acid, this does not occur; the formation of such a compound would offer considerable difficulty when the facts of re-secretion are considered; the presence of alkali in slight amounts, moreover, favours the inhibitory action of serum. A solution of serum-albumin does not cause this inhibition. It must therefore be concluded that the inhibitory action of serum is not due to the formation of an adsorption compound with bile salts, and that no such combination takes place in the blood-stream.

The bile salts must therefore be considered as being in some state, while in the plasma, other than adsorbed to the proteins—possibly uncombined: their hæmolytic action is, however, inhibited. The problems connected with this inhibition are too complicated for discussion here; the reader is referred to another paper for an account of some of them (Ponder).

Sodium taurocholate and sodium glycocholate have the properties of colloids (Ponder). This, doubtless, explains why they are excreted in such traces only. In the urine it would be expected that, as the reaction is acid, they would be adsorbed by any albumins present. This is actually the case (Wood).

While there seems no doubt that the above statements are true, so far as test-tube experiments are concerned, it is desirable to have animal experiments, in addition, to support such conclusions. A typical animal experiment may be given briefly here:

Rabbit; weight 1785 grm. Chloroform anæsthesia. The jugular vein was exposed and a cannula inserted. The urine was collected from the bladder by a tube. First a sample (1 c.c.) of normal urine was obtained. Into the jugular vein was then injected 2 c.c. of a 1 in 800 solution of sodium taurocholate in saline. After a few minutes the breathing became slightly slower, and the heart-beat slower also. The amount of urine excreted became very much diminished. No hæmoglobinuria appeared. After an interval of about half an hour, when the breathing and heart-beat had become normal, 2 c.c. of a 1 in 80 solution of sodium taurocholate in saline was injected intravenously. This quantity, it may be calculated (Ponder), when evenly distributed throughout the blood of the animal, would form a solution of taurocholate sufficiently powerful, in absence of serum, to hæmolyse 1 per cent. of the total red cells of the animal in seven minutes. A similar slight effect on the respirations and heart-beat occurred. The secretion of urine appeared to cease almost completely. After some time, however, about 1 c.c. was collected; there was no sign of hæmoglobinuria. The animal was killed, some blood drawn off, and the serum separated. The serum was clear, and showed no sign of the presence of hæmoglobin.

It may be observed that the urine collected after injection of the bile salt was more concentrated than that collected before. Any quantitative estimation of the bile salt excreted in these minute quantities of urine was, of course, impossible. On applying the reaction of Pettenkofer, according to Bertrand (Bertrand and Thomas), there was found to be no bile salts in the first (normal) specimen, and only very slight traces in the urine collected after injection of the bile salts.

These results confirm the conclusions set forth above. The action of the bile salts on the respiratory system and on the heart-beat are described elsewhere (Wells). In the experiments done in connection with this paper they were not nearly so well marked as was expected. The action on the kidney, together with other questions of importance regarding their action, is being further investigated.

CONCLUSIONS.

(1) The bile salts do not cause hæmolysis when in the blood-stream; nor does hæmoglobinuria follow their injection.

(2) This is not due to their forming an adsorption product with the albumins of the serum.

(3) Since they are colloidal in nature, they are with difficulty excreted by the kidney.

REFERENCES.

Bertrand, G., and Thomas, P.—(1920) 'Practical Biological Chemistry,' p. 146.

Croftan, A. C.—(1902) "Zur Kenntniss des intermediären Kreislaufs der Gallensäuren," Pflüger's *Archiv*, **90**, 635.

v. Noorden, C.—(1907) 'Metabolism and Practical Medicine,' **2**, 251.

Ponder, E.—(1921) "A Method for Examining the Hæmolytic Activity of Chemical Substances," *Proc. R. Soc.*, B, **92**, 285.

Stadelmann, W.—(1896) "Ueber den Kreislauf der Galle in Organismus," *Ztschr. f. Biol.*, **34**, 1.

Tapeiner.—(1878) *Sitzungsber. d. Kaiserl. Akad. d. Wissensch.*, **3**, 1.

Wells, H. G.—(1917) 'Chemical Pathology,' p. 487.

Wood, F. C.—(1918) 'Chemical and Microscopical Diagnosis,' p. 495.

INDEX TO VOL. II.

Abscesses, fixation, selective deposition of bacteria in, 276
Acetone-extracted bacteria, antigenic properties of, 131
Acriflavine, antiseptic potency of, 95
Agglutination of hæmoglobinophilic bacteria, 283
Agglutinin absorption with hæmoglobinophilic bacteria, 283
——— estimation, theory of, 214
Alexander, W., an inquiry into the distribution of the blood-groups in patients suffering from malignant disease, 66
Anæmia, secondary, reaction of the blood in, 105
Anaërobic jar, improved form of McIntosh and Fildes', 153
Andrewes, F. W., and Neave, S., the nature and systematic position of *B. paratyphosus C.*, 157
Antiseptic potency of acriflavine, 95
Antiseptics, variability of results in testing, 95
Autolysis, mechanism of, in paroxysmal hæmoglobinuria, 115

B. influenzæ, effect of blood-pigment on the growth of, 16
——— grouping of, 197
——— study of, by agglutination and agglutinin absorption, 283
B. paratyphosus C., nature and systematic position of, 157
Bacteria, acetone-extracted, antigenic properties of, 131
——— colon-typhoid group of, selective deposition in fixation abscesses, 276
——— characters of the cleavage products of, 175
——— hæmoglobinophilic, study of, by agglutination and agglutinin absorption, 283
——— influence of pure and commercial sugars, and of combined and separate sterilisation on the metabolism of, 266
Barratt, J. O. W., the theory of agglutinin estimation, 214
Benians, T. H. C., septicæmia: the selective deposition of the colon-typhoid group of bacteria in fixation abscesses,
Bennett, T. I., and Dodds, E. C., the gastric and respiratory response to meals, 58
Bile-salts, influence of, on gastric function, 70
——— relation between hæmolysis and, in the blood-stream,
Blood, physico-chemical state of sugar in the, 194
——— reaction of, in secondary anæmia, 105
Blood-groups, distribution of, in malignant disease, 66
——— relation of, to disease,
Blood-pigment, effect of, on the growth of *B. influenzæ*, 16
Blood-pressure, capillary, and the glomerular filtration theory, 205
——— ——— measurement of, in man, 1
Browning, C. H., and Gulbransen, R., the antiseptic potency of acriflavine, with considerations on the variability of results in testing antiseptics, 95
Buchanan, J. A., and Higley, E. T., the relationship of blood-groups to disease,

Cameron, G. C., see *Maitland, H. B.*
Cleavage products of bacteria, characters of, 175
Colon-typhoid group of bacteria, selective deposition in fixation abscesses,
Cowan, M. L., see *Maitland, H. B.*

Detweiler, H. K., see *Maitland, H. B.*
Dodds, E. C., see *Bennett, T. I.*
Douglas, S. R., the question of serological races of *V. choleræ* and the relation of some other vibrios to this species, 49
——— on some characters of the cleavage products of certain bacteria, with special reference to their toxicity and antigenic properties, 175
——— and Fleming, A., on the antigenic properties of acetone-extracted bacteria, 131

Dysentery immunisation in rabbits by the oral and subcutaneous methods, 256

Encephalitis lethargica, morbid histology, bacteriology and experimental pathology of, 141
EVANS, C. L., the reaction of the blood in secondary anæmia, 105

FILDES, P., the nature of the effect of blood-pigment upon the growth of *B. influenzæ*, 16
——— and MCINTOSH, J., an improved form of McIntosh and Fildes' anaërobic jar, 153
FLEMING, A., see *Douglas, S. R.*
FRASER, A. D., see *Hall, I. W.*

Gastric and respiratory response to meals, 58
——— function, influence of bile-salts on, 70
GULBRANSEN, R., see *Browning, C. H.*

Hæmagglutination and its medico-legal bearing, 26
Hæmoglobinophilic bacteria, study of, by agglutination and agglutinin absorption, 283
Hæmoglobinuria, paroxysmal, mechanism of autolysis in, 115
Hæmolysis, relation between bile-salts and, in the blood-stream, 289
Hæmolytic substances in human urine, 34
——— ——— in normal urine, 192
HALL, I. W., and FRASER, A. D., the pH range for staphylococci, 242
HIGLEY, E. T., see *Buchanan, J. A.*
HILL, L., and MCQUEEN, J., the measurement of capillary blood-pressure in man, 1
——— ——— capillary blood-pressure and the glomerular filtration theory, 205.

Immersion fluid, a new, 155
Immunity against dysentery, production of, by oral and subcutaneous methods, 256
——— production against an organism of high virulence, 239
——— ——— with saline and lipovaccines, 232
Influenza bacilli, grouping of, 197
——— vaccine, preventive effect of, 223
Isoagglutinins, theory of, 26

JENSEN, V., on picro-carmine, 103
——— a new immersion fluid, 155
JOHNSON, J. P., comparative immunity tests with saline vaccines and lipovaccines, 232
——— further studies on the production of immunity in rabbits against an organism of high virulence for the species, 239

KANAI, S., dysentery immunisation in rabbits by the oral and subcutaneous methods, 256

Lethargic encephalitis, morbid histology, bacteriology and experimental pathology of, 141
Lipovaccines, comparative immunity tests with saline and, 232

MACFIE, J. W. S., see *Yorke, W.*
MCINTOSH, J., see *Fildes, P.*
MCNALTY, A. S., the morbid histology, bacteriology and experimental pathology of *Encephalitis lethargica*, 141
MCQUEEN, J., see *Hill, L.*
MAITLAND, H. B., and CAMERON, G. C., a study of hæmoglobinophilic bacteria by agglutination and agglutinin absorption, 283
——— COWAN, M. L., and DETWEILER, H. K., spontaneous and artificial pulmonary lesions in guinea-pigs, rabbits and mice, 8
Malignant disease, distribution of blood groups in, 66
Meals, gastric and respiratory response to, 58
Metabolism of bacteria, influence of pure and commercial sugars and of combined and separate sterilisation on the,

NEAVE, S., see *Andrewes, F. W.*

ONOHARA, K., the physico-chemical state of sugar in the blood, 194
Oral immunisation of rabbits against dysentery, 256

PANNETT, C. A., and WILSON, C. M., the influence of bile-salts upon gastric function, 70
Paroxysmal hæmoglobinuria, mechanism of autolysis in, 115
PATON, D. N., and WATSON, A., the ætiology of rickets: an experimental investigation, 75
Picro-carmine, 103
PONDER, E., the occurrence of hæmolytic substances in normal urine, 192
——— the presence of hæmolytic substances in human urine, 34
——— the relation between bile-salts and hæmolysis in the blood-stream, 289
Pulmonary lesions, spontaneous and artificial, in guinea-pigs, rabbits and mice, 8

Reaction of the blood in secondary anæmia, 105
——— range for staphylococci, 242
Respiratory and gastric response to meals, 58
Rickets, ætiology of, 75

Sachs-Georgi syphilis reaction and its relation to the Wassermann reaction, 41
SCHÜTZE, H., hæmagglutination and its medico-legal bearing, with observations upon the theory of isoagglutinins, 26
Septicæmia, selective deposition of the colon-typhoid group of bacteria in fixation abscesses, 276

Staphylococci, pH range of, 242
Sterilisation of sugars, influence of combined or separate, on bacterial metabolism, 266
Sugar in the blood, physico-chemical state of, 194
——— influence of combined or separate sterilisation of, on bacterial metabolism, 266
——— influence of pure and commercial, on bacterial metabolism, 266

TANIGUCHI, T., the Sachs-Georgi syphilis reaction and its relation to the Wassermann reaction, 41

Urine, hæmolytic substances in human, 34
——— ——— normal, 192

Vaccine, influenza, preventive effect of, 223
——— saline, and lipovaccines, comparative immunity tests with, 232
V. choleræ, the question of serological races of, and the relation of some other vibrios to this species, 49
Vibrios, relation of, to *V. choleræ*, 49

Wassermann reaction, relation of, to the Sachs-Georgi reaction, 41
WATSON, A., see *Paton, D. N.*
WILSON, C. M., see *Pannett, C. A.*
WOLF, C. G. L., the comparative influence of pure and commercial sugars and of combined and separate sterilisation on bacterial metabolism, 266

YABE, S., grouping of influenza bacilli, 197
——— the preventive effect of influenza vaccine, 223
YORKE, W., and MACFIE, J. W. S., the mechanism of autolysis in paroxysmal hæmoglobinuria, 115

RB
125
J67
v. 2
cop. 2
Biological
& Medical
Serials

The British journal of
experimental pathology

PLEASE DO NOT REMOVE
CARDS OR SLIPS FROM THIS POCKET

UNIVERSITY OF TORONTO LIBRARY

STORAGE